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Ruminal degradation of ochratoxin A – *in vitro* investigations at varying rations and rumen microbial populations

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Von M. Sc. Muhammad Mobashar

aus Haripur, Pakistan

Referent: Prof. Dr. K.-H. Südekum

Korreferent: Prof. Dr. H.-W. Dehne

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Ruminal degradation of ochratoxin A – *in vitro* investigations at varying rations and rumen microbial populations

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of the requirements for the degree of

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by

M. Sc. Muhammad Mobashar

Haripur, Pakistan

Dedication

Dedicated to my beloved father, Saith Muhammad Yousaf and mother, Khanum Khan who are no more with me to see the bud of their wishes and prayers sprouting into a flower

Muhammad Mobashar

Ruminal degradation of ochratoxin A – *in vitro* investigations at varying rations and rumen microbial populations

The primary objective of the present study was to evaluate the relative contribution of different rumen microbial populations (MP) and the effect of ration on degradation of ochratoxin A (OTA), gas, short chain fatty acids (SCFA) and ammonium production at 24 h incubations using the Hohenheim gas test (HGT) *in vitro* fermentation system. Five different HGT rations (concentrate:roughage ratio (C:R) 10:90, 30:70, 50:50, 70:30 and 90:10) were used, and donor animals were fed rations with the respective ratios. The rations with the highest concentrate content were supplied with and without 1% $NaHCO_3$ (BC) (70:30BC and 90:10BC). The MP included whole rumen fluid (WRF), fungi+protozoa (F+P), bacteria+protozoa (B+P), protozoa (P) and bacteria+fungi (B+F). Protozoa numbers at 24 h, and OTA and OTα at 4, 8, 12 and 24 h were quantified. Area under the curve (AUC) and half-life for these ochratoxins were also calculated. Gas and ammonium production at 4, 8, 12 and 24 h and SCFA production at 24 h were measured.

The short average OTA half-life for whole rumen fluid of 2.6 h (range 1.3-4.5 h) demonstrates the high OTA degradation capacity of the MP (= standard HGT inoculum). MP, ration and their interaction were found to have significant effects on OTA degradation, gas and ammonium production. Among MP populations, those with bacteria (B+F and B+P) showed lower AUC values (p<0.001) for OTA (196-673 ng/ml x h) (= higher degradation capacity) and higher gas production (17.6-36.8 ml/200 mg DM) than those without bacteria (F+P and P) (701-1206 ng/ml × h and 10.8-21.4 ml/200 mg DM, respectively). In contrast, MP with P population presented higher ammonium production. Whole rumen fluid presented the lowest AUC values (146-249 ng/ml x h; p<0.05) and higher gas production (22.1-40.1 ml/200 mg DM). Ration had a quadratic effect (p=0.001) on protozoa numbers (for B+P, F+P and P). Similarly, MP and ration showed significant effect on propionate and n-butyrate production while interaction between MP and ration was only significant for propionate production. Among MP, whole rumen fluid and populations with bacteria showed higher SCFA production compared to other populations (F+P and P). It can be concluded that variations in OTA degradation, gas, SCFA and ammonium production across rations and MP were attributed to particular microbial association with the highest relevance of bacteria except for ammonium production and relative proportion of concentrate and roughage with emphasis on moderate level of C:R ratios in ration for OTA degradation.

Ruminaler Ochratoxin A-Abbau – *in vitro*-Untersuchungen zum Einfluß der Ration und verschiedener Mikrobengruppen

Das primäre Ziel dieser Arbeit war es, den relativen Beitrag der verschiedenen Populationen der Pansenmikroben (MP) und die Auswirkungen des Rationstyps auf den Abbau von Ochratoxin A (OTA), die Gasbildung, den Gehalt an kurzkettigen Fettsäuren (SCFA) und die Ammonium-Produktion mittels 24 h Inkubation im Hohenheimer Futterwerttest (HFT) als in vitro Fermentation System zu bewerten. Es wurden fünf verschiedene HFT-Rationen (Kraftfutter:Grobfutter Verhältnis (C:R) 10:90, 30:70, 50:50, 70:30 und 90:10) verwendet, und das Spendertier für den Pansensaft wurde mit der Ration im jeweiligen Verhältnis gefüttert. Die Rationen mit dem höchsten Kraftfuttergehalt wurden mit bzw. ohne 1% $NaHCO_3$ (70:30BC und 90:10BC) gefüttert. Die MP bestandenem vollständigem Pansensaft (WRF), Pilze + Protozoen (F+P), Bakterien + Protozoen (B+P), Protozoen (P) und Bakterien + Pilze (B+F). Die Protozoenzahl bei 24 h und der OTA- und OTα- Gehalt bei 4, 8, 12 und 24 h wurden quantifiziert. Es wurde weiterhin die Fläche unter der Kurve (AUC) und die Halbwertszeit für die Ochratoxine berechnet. Gasbildung und Ammonium - Produktion bei 4, 8, 12 und 24 h, sowie die SCFA-Bildung bei 24 h wurden gemessen. Die kurze, durchschnittliche OTA Halbwertzeit für den vollständigen Pansensaft von 2,6 h (Bereich 1,3 bis 4,5 h) zeigt die hohe OTA - Abbauleistung (Abbaukapazität) dieser MP (= Standard HFT Inokulum). Die MP, Ration und deren Wechselwirkungen (Interaktionen) haben einen signifikanten Einfluss auf den OTA-Abbau und die Gas- und Ammonium-Bildung. Die MP mit Bakterien (B+F und B+P) zeigten niedrigere AUC-Werte ($p < 0{,}001$) für Ochratoxin A (196-673 ng/ml x h) (= höhere Abbauleistung) und eine höhere Gasproduktion (17,6-36,8 ml/200 mg TM) als jene ohne Bakterien (F+P und P) (701-1206 ng/ml x h bzw. 10,8-21,4 ml/200 mg TM). Im Gegensatz dazu zeigt die MP mit Protozoen eine höhere Ammonium-Produktion. WRF zeigt die niedrigsten AUC-Werte (146-249 ng/ml x h, $p < 0{,}05$) und eine höhere Gasproduktion (22,1-40,1 ml/200 mg TM). Die Ration hatte einen quadratischen Effekt ($p = 0{,}001$) auf die Protozoenzahl (für B+P, P+F und P). Ebenso zeigten die MP und die Ration einen signifikanten Effekt auf die Propionat- und n-Butyratbildung, während eine Interaktion zwischen MP und der Ration nur für die Propionat-Produktion bedeutsam (signifikant) war. Bei den MP mit vollständigem Pansensaft und den anderen MP mit Bakterien zeigte sich eine höhere SCFA-Produktion im Vergleich zu anderen Populationen (F+P und P). Es kann gefolgert werden, dass Variationen im OTA-Abbau, der Protozoenzahl, der Gas-, SCFA-, und Ammonium-Produktion über Futterrationen und MP mit bestimmten mikrobiellen Kombinationen (Bakterien haben größte Bedeutung), der relative Anteil der

Kraftfutter und Grobfutter mit Schwerpunkt auf einem moderaten C:R-Verhältnis in der Ernährung eine Rolle für den OTA-Abbau spielen.

CONTENTS

ABBREVIATIONS

A. ochraceus ……..	*Aspergillus ochraceus*
ADFom……..	Acid-detergent fibre expressed exclusive residual ash
*a*NDFom…….	Neutral detergent fibre expressed exclusive residual ash
ANOVA…….....	Analysis of Variance
AOAC…….	Association of Official Analytical Chemist
app…….....	Approximately
AUC……	Area under the curve
B+F…….	Bacetria+fungi
B+P…….	Bacteria+protozoa
BC……...	Sodium bicarbonate
bw……...	Body weight
C:R…….....	Concentrate:roughage ratio
CA……..	Capsule
Conc……	Concentrate
CP……...	Crude protein
d…….....	Day
DM…….....	Dry matter
EE……...	Ether extract
F+P…….....	Fungi+protozoa
FE……...	Feed
Fig……..	Figure
GIT…….....	Gastrointestinal tract
GP……...	Gas production
HGT……	Hohenheim gas test
HPLC…….....	High performance liquid chromatography
MP…….....	Microbial population
n……..	Number
n.g……...	Not given
NFC…….....	Non-fibre carbohydrates
OTA…….....	Ochratoxin A
OTC…….....	Ochratoxin C

OTα…….....	Ochratoxin α
P……..	Protozoa
P. verrucosum……....	*Penicillium verrucosum*
RC……..	Rumen cannula
SD…….....	Standard deviation
SEM……	Standard error of mean
ST…….....	Stomach tube
TCM…….....	Temperature Control Module
TLC……	Thin layer chromatography
TMR…….....	Total mixed ration
WHO…….....	World Health Organization
WRF……...	Whole rumen fluid

LIST OF TABLES AND FIGURES

1 GENERAL INTRODUCTION

Ochratoxins, of which ochratoxin A (OTA) is the most prevalent, are secondary fungal metabolites of some toxigenic species of *Aspergillus* and *Penicillium* (Krogh, 1987; Frisvad and Samson, 1991). The natural occurrence of OTA in the food and feedstuffs is widespread (Dwivedi and Burns, 1986). OTA can contaminate feedstuffs at a level higher than 100 µg/kg (Kuiper-Goodmann and Scott, 1989) under favourable conditions, but most feedstuffs tested positive were below this level. In the tropical and subtropical areas OTA is mainly produced by *Aspergillus* species (mainly *A. ochraceus*), whereas in temperate regions such as Canada, Denmark, Germany, Sweden and United Kingdom (Jørgensen et al., 1996) *Penicillium* species are of great concern, especially *P. verrucosum* (Krogh, 1987; Frisvad and Samson, 1991).

OTA is a complex compound containing ochratoxin α (OTα) that is linked to L-ß phenylalanine through 7-carboxy group by an amide bond (Fig.1). OTA has been shown to be dominantly nephrotoxic; however, hepatotoxic, teratogenic and carcinogenic effects have also been discussed in non-ruminants (Kuiper-Goodmann and Scott, 1989). On contrary, ruminants are less sensitive to OTA due to microbial hydrolysis of OTA to its less toxic metabolite, OTα and phenylalanine by rumen microbes (Ribelin et al., 1978; Kuiper-Goodmann and Scott, 1989; Blank et al., 2004; Driehuis et al., 2010), which is considered as the principal means of detoxification of OTA in ruminants (Sreemannarayana et al., 1988). It has been reported that OTA was hydrolysed during incubation *in vitro* in rumen fluid collected from cows and sheep (Galtier and Alvinerie, 1976; Hult et al., 1976; Kiessling et al., 1984; Xiao et al., 1991a), as well as following its administration into the rumen of calves (Sreemannarayana et al., 1988) and sheep (Xiao et al., 1991b). Based on *in vitro* observations, ruminal OTA degradation capacity was estimated up to 12 mg of OTA/kg contaminated feed (Hult et al., 1976; Kiessling et al., 1984). While such *in vitro* studies mostly do not consider passage of digesta from the rumen and following absorption in the lower gastrointestinal tract (rumen bypass), they nevertheless have considerable merit in evaluating principles of ruminal degradation.

Former studies have indicated that 88-90% hydrolysis of OTA to less toxic metabolites is associated with rumen protozoa and only 10-12% with bacteria (Galtier and Alvinerie, 1976; Kiessling et al., 1984; Xiao et al., 1991a). According to previous studies degradation and systemic availability of OTA depends on concentrate:roughage (C:R) ratio in the ration, which in turn seems to be very important to maintain a active rumen microbial population (MP) with respect to OTA degradation.

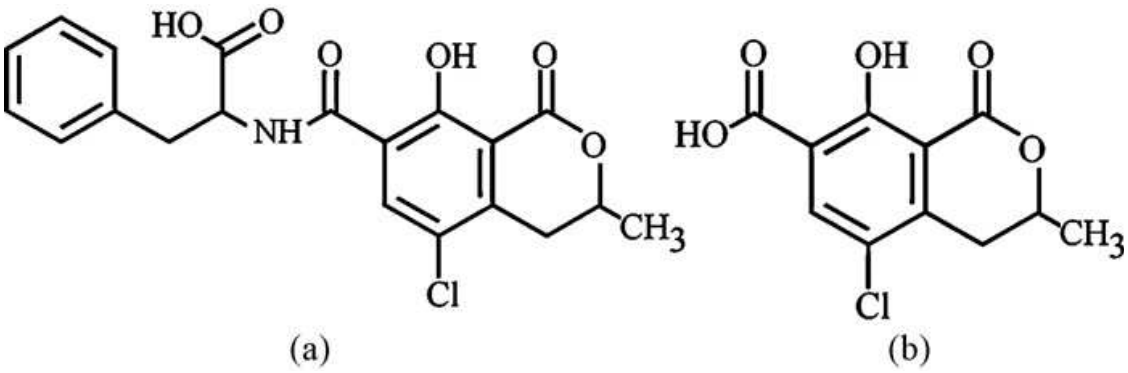

Fig. 1. Chemical structures of ochratoxin A (a) and ochratoxin α (b) (Abrunhosa et al., 2010)

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2 OBJECTIVES OF THE THESIS

This thesis was conducted in order to:

1. Analyze relevant literature on OTA in ruminants concerning its degradation by gut microbes and effects on animals.
2. Evaluate the effect of different ration types (C: R) on OTA degradation *in vitro*.
3. Estimate relative contribution of different rumen microbial populations (MP) (bacteria, protozoa, fungi) to OTA degradation *in vitro*.
4. Give particular emphasis on the role of protozoa in OTA degradation.
5. Quantify the effect of reduction of particular rumen microbial groups on organic matter (OM) degradation measured as gas production (GP) and short chain fatty acid (SCFA) production and ammonium metabolism by using the Hohenheim gas test.

A review of literature on OTA degradation by gut microbes and effects on animals is described in chapter 3. Own *in vitro* results on the contribution of different microbial populations and the influence of diet on OTA degradation are presented in chapter 4. Data on the relative contribution of different microbial co-cultures to gas production, short chain fatty acid and ammonium production from different diets in an *in vitro* fermentation system is presented in chapter 5.

3 Ochratoxin A in ruminants–a review on its degradation by gut microbes and effects on animals- review[1]

Published in Toxins

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Abstract

Ruminants are much less sensitive to ochratoxin A (OTA) than non-ruminants. The ruminal microbes, with protozoa being a central group, degrade the mycotoxin extensively, with disappearance half lives of 0.6–3.8 h. However, in some studies OTA was detected systemically when using sensitive analytical methods, probably due to some rumen bypass at proportions of estimated 2–6.5% of dosage (maximum 10%). High concentrate proportions and high feeding levels are dietary factors promoting the likeliness of systemic occurrence due to factors like shifts in microbial population and higher contamination potential. Among risk scenarios for ruminants, chronic intoxication represents the most relevant.

1. Introduction

Ochratoxin A (OTA) is a secondary metabolite of some toxigenic storage fungal species of the genera *Aspergillus* and *Penicillium* (Krogh, 1987; Frisvad and Samson, 1991). It has a widespread occurrence in foods and feedstuffs (Dwivedi and Burns, 1986), e.g., in Canada, Denmark, Germany, Sweden and United Kingdom (Jørgensen et al., 1996). In tropical and subtropical areas OTA is mainly produced by *Aspergillus* species (mainly *A. ochraceus*) whereas in temperate regions *Penicillium* species are of great importance, especially *P. verrucosum* (Krogh, 1987). Several forms of ochratoxin occur, showing varying degrees of toxicity. OTA has been shown to be dominantly nephrotoxic; however, hepatotoxic, teratogenic and carcinogenic effects are also discussed (Kuiper-Goodmann and Scott, 1989).

Negative effects of OTA can be considered on different levels of the food chain. While the most alarming would be any relevant contamination of animal products, it needs to be stated clearly that OTA level in human diets is low in general (Blank, 2002), and that the contribution of animal products to total OTA intake does not exceed 3–10% in human nutrition (EFSA, 2004). More significant proportions come from grains (54%), red wine (15%) or coffee (12%) (Blank, 2002). As desirable as further minimisation of any residual risk of OTA for the human food chain may be, the major perspective of studies on OTA in animal feeds is the reduction of negative effects on animals and their welfare and the evaluation of the level of OTA contaminated material that can be used in rations without negative effects.

It is a general experience in animal production that toxic effects of OTA are much more pronounced for non-ruminants like pigs than for ruminants. The evolution of forestomach fermentation has been regarded to have been triggered by the potential for detoxification of plant toxins (Freeland and Janzen, 1974), and the system works effectively for toxins like OTA, too: There is convincing evidence that gut microbes play the key role in OTA degradation and detoxification in ruminants. OTA is a complex compound consisting of ochratoxin α (OTα) linked through a 7-carboxy group to L-β-phenylalanine by an amide bond. Hydrolysis of this bond by microorganisms within the gastrointestinal tract leads to the far less or even non-toxic OTα and phenylalanine, which is often considered as the principal means of detoxification of OTA in ruminants (Sreemannarayana et al., 1988).

In theory, the tolerance of ruminants to the toxic effects of OTA gives them some potential as users of contaminated feeds. However, recently some doubts and discrepancies have been raised to what extent and in which amounts OTA is sufficiently detoxified by ruminants under all feeding regimes/circumstances (Höhler et al., 1999; Blank et al., 2003). It is not clear to

what extent particular microbial groups like protozoa contribute to OTA degradation and factors like the degree of contamination of typical feedstuffs need to be quantified.

Several reviews on different aspects of OTA have been published including overviews on general aspects (Schuh and Schweighardt, 1981; Marquardt and Frohlich, 1992; Höhler, 1998; Blank et al., 1999; Petzinger and Weidenbach, 2002), on OTA as a storage mycotoxin (Marquardt et al., 1990), on OTA and its mode of action (Galtier, 1998; Pfohl-Leszkowicz and Manderville, 2007; Manderville and Pfohl-Leszkowicz, 2008), on carcinogenic potential of OTA (Clark and Snedeker, 2006), on general risk assessment of OTA (Kuiper-Goodmann, 1990) or on decontamination (Valenta, 2006; Amézqueta et al., 2009).

This review is intended to contribute to a better understanding of the significance of OTA for ruminants. Since OTA contamination of diets can be expected to be mostly below a level relevant for toxic effects in ruminants, the potential of eventual aberrations from this rule will be pointed out and quantified. This appears warranted given the toxic potential of OTA. After evaluating the contamination of ruminant diets, a closer, quantitative look is depicted at factors contributing to OTA metabolism in ruminants, starting with microbial degradation of OTA. This is followed by an evaluation of systemic occurrence of OTA in ruminants and a summary of the evidence for the occurrence in ruminant products.

2. Occurrence of ochratoxin A in ruminant feeds

Given the detoxification potential of ruminants and the generally low indication for OTA occurrence in forages, it is not surprising that OTA contamination is not at the centre of mycotoxin concerns in ruminant feeds (Fink-Gremmels, 2008). However, some OTA contamination of diets can not be excluded given the variety of feeds offered to ruminants. While it can sometimes occur at concentrations high enough to cause major losses in health and performance of monogastrics, a more likely scenario for ruminants is to find a lower level of OTA occurrence in diets and only subclinical disorders related with OTA.

2.1. Concentrates

OTA has been shown to occur in various grains and other plant products throughout the world (Bauer and Gareis, 1987; Bacha et al., 1988; Bauer et al., 1988; Jelinek et al., 1989; Frank, 1991; Hald et al., 1993; MAFF, 1993). It is generally observed that concentrates are more prone to the growth of OTA producing moulds, especially in cereal feeds such as maize, barley, oats, wheat or rye and in mixed feed (WHO, 1990). OTA seems to occur more frequently and with a tendency for higher contents in mixed feeds compared to cereal grains,

although it originates dominantly from grains in this feed group, too (Thalmann, 1994; Scudamore, 1996), cited in (Valenta, 2006).

In a study on natural occurrence of OTA in feed concentrates from 13 countries including Canada and the United States until 1983, the percentage of feed that was contaminated ranged from 1 to 30% (Krogh, 1987). In Canada and the United States the contamination range was from 1 to 14.2%. OTA contamination level in animal feeds ranged by country and commodity has been highest in Northern Europe and North America according to the World Health Organization (WHO, 1990). Data of the WHO show that the countries with the highest frequency of OTA contaminated samples of animal feed were Denmark (57.6%), Canada (56.3%), and Yugoslavia (25.7%). OTA contaminations detected in each of these countries were in the range 30–27,000 μg/kg, 28–27,500 μg/kg, and 45–5,125 μg/kg, respectively.

A summary of several studies evaluating OTA levels in concentrates (particularly in a variety of cereals in Europe) is shown in Table 1, with concentration levels varying considerably. Incidence of OTA in concentrates is higher in ecological farms than in conventional farms, as has been proven in rye, wheat and barley (Czerwiecki et al., 2002), potentially due to no use of fungicides.

A survey on occurrence of mycotoxins in wheat and maize from western Romania found other mycotoxins as the major contaminants in wheat (n = 25) and maize (n = 30) (Curtui et al., 1998). OTA was only found in wheat in concentrations of 37 μg/kg in one sample. A study focusing on Brasilian dairy feeds found incidences of OTA positive samples of 25% for finished cow´s feed, 31% for maize, 22% for barley rootlets and 45% for brewers grains (Rosa et al., 2008). In dairy feeds used in 5 Sudanese farms, OTA contamination for one manufactured ration (0.22–0.61 μg/kg) and feeds like crushed sorghum (0.33–1.58 μg/kg), sunflower cake (1.59 μg/kg), wheat bran (0–0.43 μg/kg) and groundnut cake (0–0.31 μg/kg) was found (Elzupir et al., 2009). Only one of five farms was tested negative for OTA.

In a survey on Dutch dairy feeds, no OTA was found in compound feed, ensiled by-products or feed commodities (Driehuis et al., 2008a). For a variety of South-African feeds also no OTA was detected in the dairy relevant feeds wheat bran, sorghum brewers grains and a compound feed (Mngadi et al., 2008).

Table 1: Occurrence of ochratoxin A (OTA) in concentrates; table modified from (Blank et al.,1999).

Country and year	Feed	n	Positive samples	OTA content (µg/kg)	References
Poland (1975–1979)	Barley	137	19 (14%)	2–200	Juszkiewicz and Pliszcynska (1992)
	Wheat	125	15 (12%)	5–100	Juszkiewicz and Pliszcynska (1992)
	Rye	83	15 (18%)	4–200	Juszkiewicz and Pliszcynska (1992)
Denmark (1986–1992)	Wheat	520	165 (32%)	0.05–51	Jørgensen et al. (1996)
	Rye	616	256 (42%)	0.05–121	Jørgensen et al. (1996)
	Oats	92	40 (43%)	0.05–6	Jørgensen et al. (1996)
	Barley	61	17 (28%)	0.05–14	Jørgensen et al. (1996)
United States (1999)	Wheat	383	56 (15%)	0.03–31	Trucksess et al. (1999)
	Barley	103	11(10%)	0.1–17	Trucksess et al. (1999)
United Kingdom (1992)	Wheat	50	10 (22%)	1–02	Scudamore et al. (1997)
	Barley	45	12 (26%)	1–20	Scudamore et al. (1997)
Canada (1981–1983)	Wheat	440	5 (<1%)	10–51	Sinha et al. (1985)
Germany (1991–1993)	Cereals	514	10 (2%)	3–60	Richter and Schuster (1995)
Germany (1982–1987)	Barley	68	10 (15%)	0.1–206	Bauer and Gareis (1987)
	Oats	93	12 (135%)	0.1–58	Bauer and Gareis (1987)
	Wheat	64	8 (13%)	0.1–137	Bauer and Gareis (1987)
	Maize	40	3 (8%)	1.7–82	Bauer and Gareis (1987)
Netherlands (1988–1989)	Cereal grains	44	2 (25%)	6–120	Veldman et al. (1992)
	Legumes	35	10 (28%)	2–37	Veldman et al. (1992)
Egypt (1995)	Maize	54	8 (14%)	4800	Refai et al. (1996)
	Soybean	17	3 (17%)	1600	Refai et al. (1996)
	Wheat	26	2 (8%)	800	Refai et al. (1996)
Germany (1982–1987)	Mixed feed	630	89 (14%)	0.2–12	Bauer and Gareis (1987)
India (1985–1987)	Cattle cake	143	6 (4%)	n.g.	Ranjan and Sinha (1991)

n = number of samples; n.g. = not given.

2.2. Forages

While occurrence of OTA is primarily known and well established for concentrates, a review on OTA in ruminants should also consider any eventual occurrence of OTA in forages, as they normally represent the major component of ruminant diets. As a mycotoxin produced by typical storage fungi, OTA can not be expected to be present in fresh forages like fresh grass or whole-crop maize. While in silages some mould growth can occur under unfavourable aerobic conditions and OTA contaminations *via* additives can not be excluded completely, OTA presence or mould growth in silages is unlikely as long as anaerobic conditions are maintained. In consequence, most studies investigating the occurrence of OTA in silage gave no evidence for a significant occurrence: Some did not mention OTA as relevant in forages in general (FinkGremmels, 2005), others explicitly found no OTA in a large sample of Dutch maize, grass and wheat silages (Driehuis et al., 2008 a; Driehuis et al., 2008 b); in maize

silage also no significant amounts of OTA were found (Oldenburg, 1991; Garon et al., 2006; Richard et al., 2007; Richard et al., 2009). The minor OTA contents detected in one study (Oldenburg, 1991) are not quoted as positive samples by the author herself since the concentrations were within the range of detection limit.

However, individual contributions report on the occurrence of OTA in silages: values of 20–70 µg/kg were given for maize silage (Ulbrich et al., 2004), in another sample of 10 maize silages suspected to contain mycotoxins, OTA was found in all samples (mean concentration 17 µg/kg; highest content 37 µg/kg) (Kämpfe, 1999) and also another study reported on the occurrence of OTA in maize silage (Skrinjar et al., 1992). If dried forage becomes damp and fungal growth occurs, field species of fungi are likely to be gradually replaced by storage fungi such as species of *Penicillium* and *Aspergillus*. The rate at which this happens depends on the storage conditions and management practices. Insufficient drying, condensation, leakage of rain water or insect infestation can lead to further mould growth and heating (Scudamore and Livesey, 1998). The growth of storage fungi on hay can not be excluded therefore. However, the presence of *Aspergillus* or *Penicillium* alone should not be quoted as direct evidence for the presence of OTA (Auerbach and Geissler, 1992). Therefore reports on *A. ochraceus* in different forages (Le Bars and Escoula, 1973) or on *P. verrucosum* as a mould in silages and dried forages (Yiannikouris and Jouany, 2002) should prompt further investigations on OTA presence in these forages rather than to be taken as an approval of the existence of OTA in these samples.

Concerning dried forages, no evidence for the occurrence of OTA in Dutch dried forages used on dairy farms was found (Driehuis et al., 2008 b). A study on horse feeds also found no OTA in Irish and Canadian hays (n = 15) and haylages (n = 34) (Buckley et al., 2007). OTA was found only in one of 201 straw samples (Sondermann et al., 2010). In contrast, *A. ochraceus* and *P. verrucosum* were described to produce OTA at a high incidence in straw (60%) and hay (48%) (Refai et al., 1996), with astonishingly high concentrations of 8,000 (straw) and 2,800 µg/kg (hay). *Penicillium* species have a tendency to produce OTA in forages in all seasons. In a Yugoslavian study, different feed samples including forages like hay, dried lucerne and fresh lucerne were found to be contaminated with moulds at a high incidence (up to 95–100% in one year) throughout three years (Shrinjar et al., 1992). The concentration of the toxin varied from traces to 400 µg/kg.

Information cited from different sources depicted that the incidence of OTA contamination is highest in concentrates, however it may also occur in forages. Since non-ruminant animals generally are fed on concentrate-only diets, a lower daily intake of OTA can be presumed for

a ruminant compared to a non-ruminant animal. However, when high-concentrate rations or contaminated forages are used, it seems that considerable concentrations of OTA can occur in ruminant diets, too.

3. Degradation of ochratoxin A in ruminants

Two aspects need to be considered when evaluating the risk potential of OTA for ruminants: degradation of OTA to less toxic OTα and disappearance rate of OTA and metabolites from the animal and its products. In an attempt to summarize the evidence to date, at first principles and particularities of the degradation by the rumen microbial population are reviewed. In the following, studies dealing with systemic occurrence, excretion and the occurrence in animal products from ruminants are summarized.

Different approaches to minimize the toxic effects of OTA contaminated feeds exist, and they may not necessarily include enzymatic destruction of the molecule, see e.g., (Bata and Lásztity, 1999; Valenta, 2006; Blank, 2008). On the level of feeds, adsorption of the substance, heat-dependant degradation, gamma radiation or chemical treatments all have been proposed (Amézqueta et al., 2009).

Enzymatic degradation of a substance by microbes or isolated enzymes is a further option often mentioned in the context of OTA detoxification of food or feedstuffs (Abrunhosa et al., 2006), well-known to be present in the animal from classic studies (Galtier and Alvinerie, 1976; Hult et al., 1976). While other mechanisms like simply non-absorption from the gut, or low overall systemic receptiveness to a toxic substance can influence the reaction to any toxin, degradation by microbes explains much of the resistance of ruminants against OTA.

3.1. Principle of enzymatic OTA degradation

Basically, enzymatic degradation of OTA means cleavage of the amide bond into non-toxic phenylalanine and OTα (Galtier and Alvinerie, 1976; Hult et al., 1976; Patterson et al., 1981; West and Lingens, 1983; Sreemannarayana et al., 1988; Xiao et al., 1991a; Xiao et al., 1991b). Proteolytic enzymes are the most likely to develop significant OTA degrading activity. Based on the chemical structure of the OTA molecule one can speculate about the kind of proteolytic enzymes developing the highest degrading activity, and some *in vitro* studies given below have investigated the potential of OTA degradation of particular enzymes. Carboxypeptidases could be considered to be particularly effective: The enzymes cleave amide bonds at the carboxyterminal end of a peptide, and carboxypeptidase A shows

some preference for aromatic amino acids like phenylalanine as the terminal amino acid (Stryer, 1995). Both characteristics appear somewhat reflected in the molecule structure of OTA. In fact, historically carboxypeptidase A was the first enzyme shown to be effective in OTA degradation (Pitout, 1969; Doster and Sinnhuber, 1972; Deberghes et al., 1995), and is still used as a standard for activity of OTA degrading enzymes (Abrunhosa et al., 2006).

Among other proteolytic enzymes, chymotrypsin showed less and trypsin no OTA degrading activity (Pitout, 1969). Protease A and pancreatin (with protease, lipase and amylase activity) have also been shown to be effective in OTA degradation to some degree (Abrunhosa et al., 2006). Even a lipase of *A. niger* was described to have relevant OTA degrading activity (Stander et al., 2000). Lipases have been shown to have some proteolytic activity, too (Bornscheuer and Kazlaukas, 2006).

Given some effectiveness of chymotrypsin or pancreatin in OTA degradation, one could speculate to what extent degradation is performed by animal enzymes. While results on excretion of OTα in urine of non-ruminants and ruminant calves indicate the occurrence of some OTA cleavage by animal enzymes at least, empiric evidence of the high susceptibility of non-ruminant animals to OTA toxicity implies that degradation by proteolytic enzymes of the small intestine and pancreas has insufficient capacity to degrade OTA adequately. Nevertheless, the most important point is that in non-ruminant animals the enzymes start to degrade OTA only shortly before the proximal jejunum, shown to be the major site of OTA absorption in the rat (Kumagai and Aibara, 1982).

In contrast to this situation, in foregut-fermenters like ruminants, the molecule has already passed the major site of OTA degradation, the forestomach, before arriving at the major ochratoxin absorption site, the small intestine.

3.2. Degradation by different microbial populations

Inhibitory effects of OTA were present for gram-positive bacteria at a pH lower than 7 (Heller et al., 1975). However, considerable OTA degrading capacity has been described for different microbial taxa (bacteria: *Acinetobacter calcoaceticus* (Hwang and Draughon, 1994), *Lactobacillus acidophilus* and *Bifidobacterium animalis* (Fuchs et al., 2008); fungi: *Phaffia rhodozyma* (Péteri et al., 2007), *Rhizopus* spp. (Varga et al., 2005), *Aspergillus* spp. (Varga et al., 2000; Abrunhosa et al., 2002; Bejaoui et al., 2006; Abrunhosa and Venancio, 2007), *Pleurotus ostreatus* (Engelhardt, 2002) and *Trichosporon* (Schatzmayr et al., 2003). Among microbial populations involved in food production, especially those involved in beer brewing (Krogh et al., 1974; Chu et al., 1975; Baxter et al., 2001) or yoghurt production (e.g.,

Streptococcus salivarius, *Lactobacillus delbruecki* (Skrinjar et al., 1996)) have been shown to degrade OTA to some extent.

So while obviously a variety of microbes have OTA degrading capacity, gut microbial populations are probably the most well-known and among the most effective. Such populations are those found in the rat caecum (Galtier and Alvinerie, 1976; Madhyastha et al., 1992), in human faeces (Akiyama et al., 1997), in the pig gut (Schatzmayret et al., 2002) and, most prominently, in the rumen (Hult et al., 1976; Galtier and Alvinerie, 1976; Kiessling et al., 1984). As already mentioned, microbial degradation in non-ruminants like rats, pigs or humans will basically take place in the large intestine and therefore after having passed through the major absorption site. As effective as microbial degradation at this site may be, it is of insignificant relevance for the animal regarding OTA detoxification.

3.3. Degradation by rumen microbes

3.3.1. OTA degradation by the rumen microbial populations

Two lines of evidence from empiric studies have shown that the large capacity of rumen microbes for OTA degradation is closely related to the insensitivity of ruminants to the toxin: Indirectly by showing that non-ruminating calves (without or with very limited functional rumen microbes) are far more affected by negative effects of orally introduced OTA than ruminating calves (with a functional rumen microbial population) (Sreemannarayana et al., 1988), and directly by showing the quantitative transformation of OTA to OTα by rumen microbes *in vitro* or *in vivo* (Hult et al., 1976; Galtier and Alvinerie, 1976; Kiessling et al., 1984). In contrast to extensive degradation by inoculum from the three forestomach compartments (rumen, reticulum, omasum), no degradation could be shown *in vitro* with inoculum from the abomasums (Hult et al., 1976; Lerch, 1990; Müller et al., 1998).

Most studies measuring OTA disappearance from the rumen *in vivo* (in this case a combination of degradation by the microbial population, passage from the rumen and potentially some association with particulate fractions) arrive at half-lives of 0.6–3.8 h (average 2.8 ± 1.5 h), the OTA concentration being completely back to 0 after 6–13 h (Table 2). The range of OTA doses applied is considerable: From 9.5 µg/kg bw (Blank et al., 2003) to 500 µg/kg bw (Xiao et al., 1991a). As can be expected, *in vitro* studies (disappearance of OTA due to microbial degradation and potentially some association to particulate fractions) arrive on slightly higher values for half-life of 0.2–12.7 h (average 3.6 ± 3.3 h), in most studies OTA concentrations being back to zero after 6–32 h (Table 3).

Table 2: Ruminal disappearance of ochratoxin A (OTA)

OTA dose [µg/kg bw]	Diet	Ruminal OTA disappearance parameters	References
500	100% forage (hay)	Half-life 0.65 h, back to zero after app. 6 h	Xiao et al. (1991a)
500	100% concentrate	Half-life 1.30 h (30% intake), 3.38 h (100% intake); not back to zero after app. 10 h	Xiao et al. (1991a)
500	100% forage (hay)	Half-life 0.63 h, back to zero after app. 6 h	Xiao et al. (1991b)
500	100% concentrate	Half-life 2.67 h, not back to zero after 12 h	Xiao et al. (1991b)
9.5, 19.0 and 28.5	70% concentrate	Half-lives 2.60, 3.76 and 3.82 h, back to zero after app. 10–13 h	Blank et al. (2003)
14.3	70% roughage	back to zero after app. 6 h	Blank et al. (2004)
14.3	70% concentrate	back to zero after app. 13 h	Blank et al. (2004)
27.6	70% concentrate	Half-life 4.1 – 5.1 h; back to zero between 10 and 24 h	Blank and Wolffram (2009)

Table 3: Investigations on ochratoxin A (OTA) degradation during *in vitro* fermentation

OTA in rumen fluid [mg/L]	Donor animals diets	*In vitro* treatment	OTA degradation parameters	References
~0.5	rumen fluid from slaughterhouse	-	After 15 min 50% degraded; after 4 h only 5% left	Hult et a. (1976)
0.24–4.6	not given	-	0.06–0.52 mg/(h*L)	Kiessling et al. (1984)
2.5	100% hay	-	0.345 mg/(h*L)	Xiao et al. (1972a)
2.5	100% conc.	-	0.073 mg/(h*L)	Xiao et al. (1972a)
0.2	100% hay	-	Half-life 12.7 h; reduced, but not back to zero at 48 h	Özpinar et al. (1999)
0.2	80% hay	-	Half-life 4.1 h; back to zero at app. 24 h	Özpinar et al. (1999)
0.2	50% hay	-	Half-life 5.7 h; back to zero at app. 24 h	Özpinar et al. (1999)
0.2	40% hay	-	Half-life 3.9 h; back to zero at app. 24 h	Özpinar et al. (1999)
0.2	40% hay	-	Half-life 3.4 h; back to zero at app. 12 h	Özpinar et al. (1999)
0.2	40% hay	+ starch	Half-life 2.0 h; back to zero at app. 32 h	Özpinar et al. (1999)
0.2	7–8 kg DM hay, 5–6.6 kg DM conc.	-	Half-life 0.88 h; k = 0.34 h^{-1}; back to zero at app. 6 h	Müller et al. (1998)
0.2	7 kg DM hay, 4 kg DM conc.	-	Half-life 1.33 h; k = 0.23 h^{-1}; back to zero at app. 6.5 h	Müller et al. (1998)
0.2	Grass ad libitum, 3 kg DM conc.	-	Half-life 0.17 h; k = 1.75 h^{-1}; back to zero at app. 1.5 h	Müller et al. (1998)

Table 3 (continued).

OTA in rumen fluid [mg/L]	Donor animals diets	*In vitro* treatment	OTA degradation parameters	References
0.2	Grass ad libitum, 2 kg DM conc.	-	Half-life 0.51 h; k = 0.58 h^{-1}; back to zero at app. 4 h	Müller et al. (1998)
0.2	72% grass/ 18% grass hay, 10% conc.	-	k = 0.38 h^{-1} ± 0.13	Müller et al. (2001)
0.2	32% grass/ 18% grass hay, 50% conc.	-	k = 0.49 h^{-1} ± 0.07	Müller et al. (2001)
0.2	90% grass silage, 10% conc.	-	k = 0.21 h^{-1} ± 0.06	Müller et al. (2001)
0.2	50% grass silage, 50% conc.	-	k = 0.29 h^{-1} ± 0.16	Müller et al. (2001)
0.2	90% grass hay, 10% conc.	-	k = 0.22 h^{-1}± 0.07	Müller et al. (2001)
0.2	50% grass hay, 50% conc.	-	k = 0.38 h^{-1} ± 0.15	Müller et al. (2001)
0.2	40% hay, 60% conc.	-	Half-life 3.7 h; back to zero at app. 32 h	Özpinar et al. (2002)
0.2	100% hay	-	Half-life 4.5 h; back to zero at app. 32 h	Özpinar et al. (2002)
0.2	not given	+ starch	Half-life 1.9 h; back to zero at app. 32 h	Özpinar et al. (2002)
0.2	Diet with monensin	-	Half-life 20.1 h; not back to zero after 32 h	Özpinar et al. (2002)
0.07	not given	-	Half-life 3.23 h (wheat OTA); back to almost zero at 12 h; Half-life 3.06 h (crystalline OTA); back to zero at 12 h	Blank et al. (2002)

conc. = concentrate.

Non-toxic OTα is not further metabolized and would accumulate in a rumen-like environment (Lerch, 1990; Müller, 1995). On individual occasions, ochratoxin C (OTC; difference to OTA: ethyl-esterified carboxygroup of phenylalanine) (Galtier and Alvinerie, 1976) or other, unknown substances (Nip and Chu, 1979) have been described as metabolites, but at least in the former case a confusion of a protein-OTA complex with OTC can not be excluded completely (Lerch, 1990). *In vitro* results also indicate that any OTC appearing in the rumen would be metabolized to OTA at a high rate, and therefore finally be degraded to OTα (Lerch, 1990).

According to several studies, OTA degradation can be considered a first-order reaction, which means that the reaction is dependant on the concentration of the substrate mainly. No blocking effect of developing OTα has been shown. The existence of first-order kinetics is explained by the fact that OTA has to pass into the cell to be degraded, and that any such concentration-

dependant influx can be considered a first-order process (Müller et al., 1998). It has been suggested that little extracellular proteolytic activity contributes to OTA degradation. This is supported by the lack of comprehensive OTA degradation activity in the fluid phase of inoculum of *in vitro* studies investigating the different rumen microbial groups concerning their OTA degrading capacity (Galtier and Alvinerie, 1976; Kiessling et al., 1988; Xiao et al., 1991a). The fact that OTA follows monoexponential decay also implies that some low concentration of OTA will stay in the rumen fluid longer than what might be expected from half-life alone.

3.3.2. OTA degradation capacity of different microbial groups

Different groups of microbes (protozoa, bacteria or fungi) could be considered to contribute in metabolising OTA. The predominant microorganisms in the rumen are generally capable of utilizing a variety of substances, however there are contradictory studies that showed that rumen microflora is very limited and specific in toxin degradation capacity and activity and requires specific substrate for growth and as such fill a unique ecological niche.

Table 4: Ochratoxin A (OTA) degradation by different fractions of rumen microbes

OTA in rumen fluid	Microbial fraction	Degradation rate OTA	References
~12.5 mg/L	Protozoa[1]	54% degraded after 24 h	Galtier and Alvinerie (1976)
~12.5 mg/L	Heavy bacteria[1]	13% degraded after 24 h	Galtier and Alvinerie (1976)
~12.5 mg/L	Light bacteria[1]	No degradation after 24 h	Galtier and Alvinerie (1976)
0.2 mg/L	Protozoa + heavy bacteria (200 g; 10 min)	app. 90% degraded after 4 h	Kiessling et al. (1988)
0.2 mg/L	Bacteria (supernatant)	app. 10% degraded after 4 h	Kiessling et al. (1988)
0.2 mg/L	Rumen fluid minus protozoa[2]	app. 15% degraded after 4 h	Kiessling et al. (1988)
1250 mg/L	particulate fraction (centrifugation 10 min at 150 g)	201 µg/(h*L) (hay diet)	Xiao et al. (1991a)
1250 mg/L	supernatant centrifugation	17 µg/(h*L) (hay diet)	Xiao et al. (1991a)
0.2 mg/L	Protozoa fraction	Half-life 2.44 h; back to zero at app. 32 h	Özpinar et al. (2002)
0.2 mg/L	Bacteria fraction	Half-life 99.4 h; not back to zero after 32 h	Özpinar et al. (2002)
Not given	Rumen bacteria	Able to degrade OTA	Schatzmayr et al. (2002)

[1] Fractionated centrifugation for 5 min at 166 g for protozoa, for 10 min at 1,500 g for heavy bacteria and for 20 min at 20,000 g for light bacteria. [2] Treated with dioctyl sodium sulfosuccinate

Concerning OTA degrading capacity of different rumen microbes (Table 4), from the beginning protozoa were found to be the most active microbes in OTA degradation (Galtier and Alvinerie, 1976), as confirmed repeatedly (Kiessling et al., 1988; Xiao et al., 1991a; Özpinar et al., 2002).

The overall capacity of rumen fluid to degrade OTA was shown to decrease if rumen fluid is collected shortly after feeding (Kiessling et al., 1984) and is related to the variation in the composition of protozoa due to change in dietary composition and feeding time. However, investigations on the contribution of different groups of protozoa (*Entodiniinae, Diploiniinae, Ophryoscolecinae and Isotrichidae*) gave no indication for clear differences between the OTA degradation capacities of these groups (Lerch, 1990).

Some information indicates that gut bacteria can also have considerable OTA degrading capacity. Degradation of OTA by microbes of the hindgut (large intestine/caecum) of rats, pigs and humans (all known to lack a protozoal population) shows that gut bacteria are also capable of OTA degradation.

Results of some studies indicated significant capacity for OTA degradation of the rumen bacterial fraction, too (Kiessling et al., 1988; Schatzmayr et al., 2002).

The role of fungi has not been investigated comprehensively. When considering their high proteolytic capacity (Wallace and Joblin, 1985), and the fact that cellulose increased OTA degradation (Müller, 1995), fungal OTA degradation activity in the rumen could be present (Müller et al., 1995).

3.3.3. Influence of ration composition on OTA degradation

Any influence of ration composition on ruminal OTA degradation will most likely be mediated by its influence on the microbial population. Given their prominent role, influence on protozoa is most interesting. Protozoa populations can be influenced by dietary factors like ration composition, feeding level and frequency of feeding, mediating important variables of the rumen habitat like available substrates, pH or turnover rate (Franzolin and Dehority, 1996). Obvious interrelations between these variables exist and complicate simple conclusions: High concentrate rations will lower pH much less when being fed at low feeding level only, and any lack of particles to attach may have less consequences for protozoa when overall turnover rate of the rumen is low.

It appears that rations with 40–60% concentrate promote the highest protozoa density (Dehority and Orpin, 1988). In rations of low concentrate level energy limits the population at some point. In rations with a high concentrate level low pH is likely to occur due to fast short

chain fatty acid production and little stimulation of chewing and therefore limited buffer influx *via* salivation. The pH in the rumen can vary from more than 7.0 on a roughage diet to less than 5.0 on high grain diets (Russell and Dombrowski, 1980; Erfle et al., 1982; Erdmann, 1988), the effect of concentrate on the pH being dependant of the feeding level and adaptation of the animal to the diet. When dairy cows are consuming a total mixed ration (TMR) twice a day, the ruminal pH may range from 5.5 to 6.5 and on fresh high quality pasture, the pH may range from 5.6 to 6.8 within a 24 hour period (de Veth and Kolver, 2001). Ruminal pH generally continues to decline 4–6 hours after feeding (Lindberg, 1981; Madsen and Hvelplund, 1988). Total duration of pH drop below certain thresholds are considered to be most significant for the effects of pH changes (Zebeli et al., 2008). Since protozoa limit being washed out from the rumen actively by attaching to larger particles, a reduction of such structures to attach and therefore to limit outflow may be a constraint for the protozoa population, too. The percentage of *Entodinium* usually increases as the amount of concentrate in the ration increases, and most of the time results in an *Entodininium*-only fauna or complete disappearance of the protozoa.

Ration influences OTA degradation primarily *via* its influence on the composition of the rumen microbial population. In fact, when comparing a diet with an extreme level of concentrate of 100% with a forage-only diet, much higher microbial OTA degradation activity in the rumen of hay fed sheep was found (Xiao et al., 1991a). For less extreme diets (70% concentrate and 30% forage and vice versa), an *in vivo* study in sheep also found slower OTA degradation in the high-concentrate diet indicating the importance of concentrate to forage ratio in this respect (Blank et al., 2004). *In vitro* studies are supporting this view: In accordance with the idea of maximal protozoa populations at intermediate concentrate levels, a higher OTA degradation rate in a diet consisting of 40% roughage and 60% concentrate compared to a diet consisting of 100% hay was found (Özpinar et al., 1999). By adding starch, ruminal OTA halftime was 1.9 h while it increased to 4.5 h by the addition of cellulose (Özpinar et al., 2002). Also in another study a higher proportion of concentrate (50% *vs.* 10% DM) resulted in a higher degradation of OTA (Müller et al., 2001).

Interestingly it was shown *in vitro* that diets high in fresh grass improved OTA degradation compared to grass hay or grass silage based diets (Müller et al., 1998; Müller et al., 2001). Capacity of forages to promote OTA degradation was ranked to be grass > grass hay > grass silage (Müller, 1995). It is not clear whether this is due to a more prominent proteolytic population (as indicated above), or due to a generally higher microbial density on the grass based diets.

3.3.4. Further aspects of rumen microbial degradation of OTA

Some influence of dosing in relation to feeding time has been reported by two independent studies, consistently finding lowest activity directly after feeding, and higher activity before and 4–6 h after feeding (Sreemannyrayana et al., 1988; Xiao et al., 1991a). While fluctuations in the microbial population have been proposed as an explanation, the long generation time of protozoa (6–15 h for *Entodinia*, and 24–48 h for other groups; (Van Soest, 1994)) makes them rather unlikely to undergo population changes in such short time frames. At least contributing to a decrease in activity is the dilution of protozoa concentration due to an increased flux of water into the rumen after a meal, which is caused by increased drinking and salivary activity and osmolytically driven water-flux into the rumen *via* the rumen wall. Some competition of OTA with postprandially abundant food protein for proteolytic enzyme capacity should also be considered to contribute to this effect. As could be expected from investigations on other catabolic microbial activity, rumen fluid of cattle and sheep did not show a difference in OTA degradation (Kiessling et al., 1984).

Concerning any interpretation of OTA disappearance from the rumen fluid it must also not be forgotten that some association of OTA with all kinds of particles (e.g., bacteria, fungi and food particles) can occur, which may also detract OTA from the fluid phase to some extent (being still available for the host animal). As mentioned, degradation measured *in vitro* (non continuous culture!) contrasts slightly from disappearance *in vivo*, since in the latter situation, rumen passage will contribute to OTA turnover depending on the level of feed intake. Based on fluid passage rates from the rumen, a rumen bypass of 2 up to 6.5% of ingested OTA for sheep with maintenance requirements was estimated for the first hour of incubation (with a maximum of 10% after 4 h) (Müller, 1995). Higher feeding levels increase ruminal passage rate, thereby increasing ruminal bypass proportion.

An influence on digestibility as stated in some studies (Blank et al., 2003) may be best explained to be a result of the diarrhea induced by the mycotoxin and not by significant direct negative effects on microbes. However, other studies found no (*in vivo*, (Höhler et al., 1999)) or only slight indications (*in vitro*, (Abdelhamid et al., 1992)) for a negative influence of OTA on digestibility. Using four toxin concentrations of 5.0, 20.0, 200 and 1,400 µg/L of incubation medium, a significant decrease of *in vitro* digestibility was found only at the highest concentration (Pettersson and Kiessling, 1976).

4. Systemic occurrence and excretion of OTA in ruminants

4.1. OTA in pre-ruminating ruminants

Compared to the plethora of reports on diverse OTA-caused pathological lesions in monogastric animals such as pigs Krogh et al., 1974; Rutqvist et al., 1978; Krogh, 1987; Jelinek et al., 1989) or poultry (Hamilton et al., 1982; Dwivedi and Burns, 1986; Kuiper-Goodmann and Scott, 1989), there is comparatively little evidence and reports on pathological effects of OTA in ruminants. Acute toxicity can be demonstrated for young ruminants (functional monogastrics) without developed rumen and therefore lacking a functional microbial population in their guts (Table 5).

Calves died within 24 h from single OTA doses of 11,000 and 25,000 μg/kg bw (n = 2, 5 weeks old) (Ribelin et al., 1978) or from a dose of 4,000 μg/kg bw (n = 2, 3 weeks old) (Sreemannarayana et al., 1988). At lower doses (1,000 and 500 μg/kg bw; n = 2 each), one of two or no calves died in the latter study. Calves even survived a 30 d OTA dosage of up to 2,000 μg/kg bw per day. However they showed some serious signs of intoxification (polyuria and general depression, plus some kidney degeneration even at the lowest dose of 100 μg/kg bw) (Pier et al., 1976). Since the calves used in this study were already 2–3 month old and received a pelleted diet 1–2 month before the start of the trial, some development of rumen flora may already have started, and further development due to the start of hay feeding at the same time as OTA dosing reduced negative effects of OTA on these animals. In fact, the authors explain the improvement of the situation during the 30 day dosage period by the increased detoxification capacity of the developing rumen microbial population.

4.2. OTA in functional ruminants

The fact that even rather young animals with developed rumen have been shown to be much less affected by OTA than pre-ruminant calves (Sreemannarayana et al., 1988) indicates the significance of ruminal degradation of OTA for detoxification. This is underlined by results of intravenous dosing of OTA (Table 6). As can be expected, such total circumvention of the rumen results in significant toxicity in adult ruminants, up to death of sheep at a single dose of 1,000 μg/kg bw (Munro et al., 1973), which can be considered much less critical when given orally (Ribelin et al., 1978). However, sheep appeared to be relatively little affected by a single intravenous dose of 200 μg/kg bw (Xiao et al., 1991b).

There are some old anecdotal and sometimes speculative reports on potential harmful effects of dietary OTA on adult ruminants from agricultural practice. An example are reports on the

potential occurrence of abortions in dairy cattle after consumption of mouldy lucerne/grass forage (Still et al., 1971; Still, 1973).

The death of 15 of 30 cows due to ochratoxicosis when these animals were fed an improperly ensiled oats-lucerne mixture was reported (Vough and Glick, 1993). Negative consequences ranging from general depression, fever diarrhoea and uraemia until death (113 out of 11,500 animals) in connection with affection of kidneys in steers, cows and ruminating calves after consuming OTA-contaminated diets were mentioned on another occasion (Lloyd and Stahr, 1980; Lloyd and Citrinin, 1980), both as cited in (Scudamore and Livesey, 1998).

From the background of these reports, a further look on animals with a functional rumen seems warranted irrespective of the evidence of comprehensive OTA degradation in the rumen. In a study focusing on OTA analytical methods, losses of cattle after the consumption of mouldy wheat contaminated with OTA at a level of 100 µg/kg were mentioned (Scott et al., 1970), implying a potential influence of OTA. It has to be added here that in studies reporting on farm outbreaks of mycotoxin intoxication, pathological effects can well be due to other mycotoxins like citrinin, as has been explicitly stated by some authors (Scott et al., 1970; Lloyd and Citrinin, 1980). OTA is often associated with other mycotoxins like citrinin, penicillic acid or hydroaspergillic acid (Ribelin et al; 1978), contributing significantly or even dominantly to effects on animals and microbes if present in sufficient amounts. This factor also needs to be taken into account when using naturally OTA contaminated feeds in more defined trials.

Table 5: Effects and systemic occurrence of ochratoxin A (OTA) in preruminant calves

OTA dose [µg/(kg bw*d)]	Dosing method	Duration	Animal age	n	Clinical effects; systemic presence of OTA + OTα	Excretion of OTA + OTα in urine and faeces	Detection limit chemi-cal analysis	Diet	References
500	ST	Single dose	16–21 d (60 kg)	2	Both calves survived; no suppressive effect on feeding; serum contents app 0.2–0.4 µg OTA/mL (still 5 d after dosing)	Dose recovery 97%; 88% as OTα and 3.4% as OTA in urine; 9.2% as OTA in faeces	50 ng/mL HPLC	Milk	Sreemannarayana et al. (1988)
1000	ST	Single dose	10–15 d	2	1 calf dead within 12 h, 1 calf survived; labored beathing, severe diarrhea, prostration; cessation of feeding for 4 h in the surviving calf	-	-	Milk	Sreemannarayana et al. (1988)
4000	ST	Single dose	10–15 d	2	Dead within 24 h; labored beathing, severe diarrhea, prostration; refused to feed for 4 h	-	-	Milk	Sreemannarayana et al. (1988)
11000	ST	Single dose	35 d	1	Dead within 24 h (epicardial hemorrhages)	-	-	Not given	Ribelin et al. (1978)
25000	ST	Single dose	35 d	1	Dead within 24 h (epicardial hemorrhages)	-	-	Not given	Ribelin et al. (1978)
100, 500	CA	30 days	2 month	1+1	Polyuria on app. day 20; tended to revert to normal at the end of experiment; necropsy: pale kidney, mild enteritis, mild tubular kidney degeneration	-	-	Roughage feeding at start of experiment	Pier et al. (1976)
1000, 2000	CA	30 days	2 month	1+1	Polyuria, depressed (on day 14 in the low and day 2 in the high dose), dehydrated; symptoms tended to revert to normal at the end of experiment); necropsy: pale kidney, mild enteritis, mild tubular kidney degeneration	-	-	Shift milk replacer to pellets first month of age; roughage feeding at start of experiment	Pier et al. (1976)

n = sample size; ST = *via* stomach tube; CA = orally in capsule; µg/(kg bw*d) = µg OTA per kg body weight and per day.

Table 6: Effects and systemic occurrence of ochratoxin A (OTA) when applied intravenously (single dose).

OTA dose [µg/(kg bw*d)]	Animal, age, bw	n	Clinical effects; systemic presence of OTA + OTα	Excretion of OTA + OTα in urine and faeces	Detection limit/ chemical analysis	Diet	References
200	Sheep, adult, 50 kg	4	Seemed normal; urine volume increased; maximal 4 µg OTA/mL in blood serum	Dose recovery (6 d) 57–61%; excretion in urine 93% as OTA and 3.2% as OTα, in faeces 4.4% as OTα	50 ng/mL, HPLC	Hay	Xiao et al. (1991b)
250	Cattle, not lactating or pregnant, 400 kg	1	Not mentioned	Only OTA in urine, no OTα	TLC	Dairy ration	Still (1973)
250	Calves, 19–20 d, 44 kg	2	1 dead after 31 h; 1 survived; no cessation of feed intake; serum OTA from 3.0 to 0.1 µg OTA/mL during 5 d	Dose recovery 70%; excretion in urine 36% as OTA; in faeces 64% as OTA; no OTα in urine or faeces	50 ng/mL, HPLC	milk at 10% bw (over night fast)	Sreemannarayana et al. (1988)
1000	Sheep, 135 days pregnant	2	Dead after 12 and 24 h; pulmonary congestion and edema; liver necrosis; serum OTA from 7–8 to 1–3 µg OTA/mL during 12 h	-	not given	not given	Munro et al. (1973)

n = sample size; TLC = thin layer chromatography; HPLC = high pressure liquid chromatography; n.g. = not given.

Obviously only studies dosing OTA orally represent the situation of OTA in functional ruminants in a realistic way. In this respect it can be considered rather irrelevant whether the dosage occurred orally (deliberate intake or *via* oro-ruminal probe) or intraruminally (*via* a rumen fistula) and at least secondary whether OTA was included in the normal feed or as capsule and whether the crystalline substance or naturally contaminated feeds were used (Lerch, 1990; Blank et al., 2002). As a rough estimate of the OTA contamination a ruminant may have to face in a practical situation, daily doses of a size of approximately 2.0 µg/kg bw (assumptions: 600 kg bw, 20 kg DM intake; OTA contamination of diet 50 µg/kg DM) appear possible (although representing an over- rather than an underestimation of the average situation) while a daily dose of 20 µg/kg bw rather represents the maximal imaginable dose if an extreme situation is assumed (500 µg OTA/kg DM). The studies summarized in Table 7 (application of OTA >14 days, starting at a dose of 9.5 µg/kg bw) represent the practical situation much more than studies dosing OTA for a short period but at a rather high dose (Table 8; application of OTA from 1–6 days).

4.2.1. Pathological findings and systemic occurrence

The lack of a general protocol (e.g., whether signs were actually searched for, or only noticed by chance when very clear) complicate comparison of studies, but conclusions can still be drawn from the information available to date. Clear signs of discomfort and starting intoxification (polyuria, reduced feed intake) are first reported at repeated doses of 225 µg/kg bw (Höhler et al., 1999) or at a single dose of 500 µg/kg bw (polyuria; (Xiao et al., 1991b)) for sheep. At a single, but very high dose of 13,300 µg/kg bw a cow showed severe clinical signs like difficulties in arising, diarrhoea, anorexia and cessation of milk production, the latter only recovering to one third of normal during this lactation (Ribelin et al., 1978). In the same study, an adult goat died within 5–6 days after daily oral OTA doses of 3,000 µg/kg bw. This is not to say that such effects can not occur at lower levels but rather that one can expect such effects at the OTA doses used in these studies.

Little work has been published on changes of the target tissues of OTA like kidney or liver. While only minimal microscopic kidney changes were reported in goats fed a high OTA dose for 14 days (Ribelin et al., 1978), another study did not find pathological kidney changes at practically relevant OTA levels and dosage periods (Patterson et al., 1981).

While such information could be interpreted as a justification of considering OTA as a mycotoxin of no relevance and completely uncritical in ruminants, repeated reports on the systemic occurrence (like in blood and in urine) not only of the harmless OTα metabolite, but

also of OTA show that detoxification is less than might have been expected. Even repeated doses of 9.5 µg/kg bw (Blank et al., 2003) led to OTA in blood serum and urine of sheep. Overall, transfer of OTA into blood is linearly increasing with the dose of OTA (Blank et al., 2003).

Table 7: Systemic occurrence of ochratoxin A (OTA) and ochratoxin α (OTα) in functional ruminants after oral dosing over >14 days

OTA dose [µg/(kg bw*d)]	Study duration	Animal, age, bw	n	Clinical and pathological effects; systemic presence of OTA + OTα	Excretion of OTA + OTα in faeces and urine	Detection limit/ chemical analysis	Diet	References
9.5	29 d	Sheep, 1 year, 39 kg	3	No overt illness; food intake not influenced; blood serum OTA 1.5–6.0 ng/mL, OTα 0.4–0.8 ng/mL	Dose recovery 80% (7 d); 1.9% OTA and 20.4% OTα in faeces, 7.8% OTA and 70% OTα in urine	0.2 ng/mL (HPLC)	70% conc. + 30% grass silage	Blank et al. (2003)
14	31 d	Sheep, adult, 58 kg	3	No overt illness; food intake not influenced; blood serum OTA 2–4 ng/mL	Dose recovery 81% (7 d); 1.5% OTA and 11.2% OTα in faeces, 4.4% OTA and 82.9% OTα in urine	0.2 ng/mL (HPLC)	70% roughage	Blank et al. (2004)
14	31 d	Sheep, adult, 58 kg	3	No overt illness; food intake not influenced; blood serum OTA 4–9 ng/mL (tendency to accumulate).	Dose recovery 78% (7 d); 0.9% OTA and 18.5% OTα in faeces, 5.8% OTA and 75% OTα in urine.	0.2 ng/mL (HPLC)	70% conc.	Blank et al. (2004)
14.7–16.5	87 d	Cattle, 12 weeks, 80 kg	6	No liver, kidney, skeletal or heart muscle damage; 3 calves with some OTA in kidney	no OTA in urine but some OTα	-	1.5 kg hay + 1.5–2.7 kg conc.	Patterson et al. (1981)
12.0–16.0[1]	87 d	Cattle, 12 weeks, 80 kg	6	No liver, kidney, skeletal or heart muscle damage; 2 calves with some OTA in kidney	no OTA in urine but some OTα	-	1.5 kg hay + 1.5- 2.7 kg conc.	Patterson et al. (1981)
~18	77 d	Cattle, adult, app. 400 kg	2	Clinically normal; lesions on kidneys, subacute interstitial nephritis; some OTA detected in kidneys of one animal; no OTA or OTα in milk, muscle or liver reported	No OTA or OTα detected in urine.	-	9 kg hay + 10 kg conc.	Shreeve et al. (1979)
19	29 d	Sheep, 1 year, 39 kg	3	No overt illness, food intake not influenced; blood serum OTA 4.6–12.4 ng/mL, OTα 0.7–2.3 ng/mL	Dose recovery 78% (7 d); OTA 7.7% in urine and 1.9% in faeces; OTα 20.7% in faeces and 70% in urine	0.2 ng/mL (HPLC)	70% conc.+ 30% grass silage	Blank et al. (2003)
22	28 d	Sheep, adult, 66 kg	4	No overt illness; food intake not influenced; normal weight gain; blood serum OTA 8.2–10.8 ng/mL, OTα 2.0–3.4 ng/mL	Dose recovery 75%; OTA 5.1% in urine and 1.1% in faeces; OTα 13% in faeces and 81% in urine	HPLC	70% conc.+ 30% hay	Höhler et al. (1999)
28.5	29 d	Sheep, 1 year, 39 kg	3	No overt illness; food intake not influenced; blood serum OTA 6.4–18.2 ng/mL, OTα 0.7–1.6 ng/mL	Dose recovery 74% (7 d); OTA 12% in urine and 3.4% in faeces; OTα 36% in faeces and 49% in urine	0.2 ng/mL (HPLC)	70% conc. + 30% grass silage	Blank et al. (2003)
55	28 d	Sheep, adult, 66 kg	4	No overt illness; food intake not influenced; normal weight gain; blood serum OTA 67.0–111.7 ng/mL, OTα 12.0–18.5 ng/mL	Dose recovery 84%; OTA 4.8% in urine and 0.59% in faeces; OTα 16% in faeces and 91% in urine	HPLC	70% conc. + 30% hay	Höhler et al. (19991)
225	14 d	Sheep, adult	n.g.	Reduction in feed intake (toxicosis); blood serum OTA 36 ng/mL, OTα 15 ng/mL	-	HPLC	70% conc. + 30% hay	Höhler et al. (19991)
1000, 2000	14 d	Goat, adult, 59 kg	1,1	No clinical signs besides diarrhoea and polyuria promoting haemoconcentration (urea N up, minimal microscopic kidney changes)	-	-	Lucerne hay + conc.	Ribelin et al. (1978)

n = sample size; n.g. = not given; conc. = concentrate; HPLC = high pressure liquid chromatography; [1] plus aflatoxin.

Table 8: Systemic occurrence of ochratoxin A (OTA) and ochratoxin α (OTα) in functional ruminants after oral dosing ≤6 days

Dose [µg/(kg bw*d)]	OTA appl.	Duration	Animal, age, bw	n	Clinical and pathological effects; systemic presence of OTA + OTα	Excretion of OTA+OTα in faeces and urine	Detection limit/method	Diet	References
22 (4 d) + 55 (2 d)	FE	4+2 d	Sheep, adult, 50 kg	1	Not commented on; no OTA and OTα in serum 1 h after dose	-	n.g.	Not given	Kiessling et al. (1984)
27.6	FE	Single dose	Sheep, adult, 89 kg	6	No overt illness; blood serum OTA max 14.4 ng/mL	Dose recovery 86%; OTA 6.5% in urine and 3.7% in faeces; OTα 34% in faeces and 56% in urine	0.2 ng/mL HPLC	70% conc. + 30% grass silage	Blank et al. (2003); (Wolffram, 2009)
200	FE	4 d	Cattle, lactating, not pregnant, 500 kg	1	No overt clinical signs; delivery of normal calves; no OTA and OTα up to 200 µg/kg DM in milk (back to zero 1.5 d after last dose)	No OTA and up to 8 µg/mL OTα in urine	TLC	Dairy cattle ration	Ribelin et al . (1978) ; Shreeve et al. (1979)
250	ST	Single dose	Cattle, not pregnant or lactating, 400 kg	1	No overt clinical signs	No OTA and up to 2 µg/mL OTα in urine	TLC	Dairy cattle ration	Shreeve et al. (1979)
500	CA	Single dose	Goat, adult, 45 kg	2	Not commented on; 6% in milk and 2% in serum (in the latter 3 dominantly as undetermined metabolites)	Excretion of OTA dose: >90% within 7 days, excretion 54% in faeces (dominantly as OTA), 38% in urine	TLC	Hay	Nip and Chu (1979)
500	CA	Single dose	Goat, adult, 45 kg	2	Not commented on; 1.5% and 0.5% of total dose found in liver and kidney 6 h after dose	-	TLC	Hay	Nip and Chu (1979)
500	RC	Single dose	Sheep, adult, 60 kg	2	No overt illness; notion of increased urine volume; in blood serum OTA up to 400 ng/mL at 100% intake and 150 ng/mL at 30% intake 4 h after dose	Dose recovery 67%; OTA 1.2–2.8% in urine and 0.28–0.29% in faeces; OTα 7.6–18% in faeces and 81–89% in urine	HPLC	100% grain	Xiao et al. (1991b)
500	RC	Single dose	Sheep, adult, 60 kg	2	No overt illness; notion of increased urine volume; in blood serum OTA up to 100 ng/mL 4 h after dose	Dose recovery 58%; OTA 0.56% in urine and 0.93% in faeces; OTα 24% in faeces and 75% in urine	HPLC	100% hay	Xiao et al. (1991b)
500	RC	Single dose	Sheep, adult, 20 kg	4	No overt illness; area-under-the-curve (AUC) blood serum OTA 6495 (ng*h/mL) and OTα 196	-	HPLC	100% grain	Xiao et al. (1991b)

					(ng*h/mL)				
500	RC	Single dose	Sheep, adult, 20 kg	4	No overt illness; area-under-the-curve (AUC) blood serum OTA 1456 (ng*h/mL) and OTα 494 (ng*h/mL)	-	HPLC	100% hay	Xiao et al. (1991b)
750	ST	5 d	Cattle, 3 mon pregnant, not lactating, 600 kg	1	Delivery of normal calves; in milk no OTA, but traces of OTα	Traces of OTα in urine	TLC	Dairy cattle ration	Ribelin et al. (1978) ; Still (1973)
1660	ST	5 d	Cattle, 6 mon pregnant, lactating, 600 kg	1	Delivery of normal calves; in milk OTA app. 100 µg/kg DM on day 3,4 and 5; back to zero 2 days after dose; OTα 750 µg/kg DM on day 1–6	Traces of OTα in urine	TLC	Dairy cattle ration	Ribelin et al. (1978; Still (1973)
2000	ST	Single dose	Cattle, 46–69 d, 68–100 kg	4	No overt illness; in blood serum OTA 2.0–0.1 ng/mL (decrease over 5 d) and OTα 0.1–0.2 ng/mL	Dose recovery 92%; excretion as OTA 0.4% in urine and 1.9% in faeces; as OTα 82% in urine and 16% in faeces	50 ng/mL	Barley + hay	Sreemannarayana et al. (1988)
3000	ST	5 d	Goat, adult, pregnant, 59 kg	1	Dead after 5 days; diarrhea, dehydration; no gross lesions; microscopical lesions confined to centrolobular swelling of liver	-	-	Lucerne hay + conc.	Ribelin et al. (1978)
13300	ST	Single dose	Cattle, lactating, 6 months pregnant, 600 kg	1	Difficulty in arising, diarrhea, anorexia 1–4 d after dosing; drastic reduction of milk production; in milk OTA up to 640 µg/kg DM, OTα 4500 µg/kg DM after one day	Traces of OTα in urine	TLC	Dairy cattle ration	Ribelin et al. (1978); Still (1973)

FE = orally in feed; ST = *via* stomach tube; RC = rumen cannula, CA = orally in capsule; appl. = application; HPLC = high pressure liquid chromatography; TLC = thin layer chromatography

4.2.2. Excretion via urine and faeces

Apparently, intravenous dosage of OTA seems to circumvent all sites of degradation of OTA to OTα, since in two studies, 90–100% of the dose was found to be excreted as OTA (Still, 1973; Xiao et al., 1991b). It can be safely concluded that OTA degradation is very limited after absorption from the gut. The main route of excretion of OTA in functional ruminants dosed orally is as its metabolite OTα *via* urine. Interestingly, in a study using young ruminants most of the OTA dose was found to be excreted in the form of OTα (Sreemannarayana et al., 1988). This could stem from degradation of OTA by animal or microbial enzymes. Potentially, the calves investigated already had some amylolytic population, which would also develop significant proteolytic activity, even if there is not yet significant fibrolytic activity present.

4.2.3. Particularities influencing OTA toxicity and degradation in ruminants

The systemic occurrence of OTA in ruminants despite the microbial population actively degrading the toxin can best be explained by the fact that the rumen functions comparable to a mixing chamber, which allows some outflow (bypass) of any substrate entering the rumen (Blank et al., 2003), irrespective of the measured long mean retention times. OTA escaping ruminal degradation will readily be absorbed in the lower digestive tract, just as in non-ruminants.

Several factors have been discussed in their influence on OTA toxicity in ruminants, most of them connected to ruminal degradation. A significant influence of diet (mostly quantified as concentrate to roughage ratio) has been indicated by two studies (Xiao et al., 1991b; Blank et al., 2004), pointing to a more comprehensive bioavailability of OTA in concentrate based diets. Besides influencing the microbial population (as outlined above in more detail), a low rumen pH will also increase the occurrence of OTA in its nondissociated ("protonated") form, which will also facilitate absorption of OTA from the rumen and therefore systemic availability of the toxin (Blank et al., 2003). Based on the consideration that general proteolytic enzymes catalyze the cleavage responsible for OTA detoxification, a signifcant improvement of degradation capacity *via* adaptation of the microbial population does not seem probable, e.g., in contrast to fibre digestion.

There are further relevant aspects for systemic OTA metabolism in ruminants. Biliary secretion (like demonstrated in other taxa like rats) has been postulated for OTA (Xiao et al., 1991b), explaining repeated peaks in blood concentrations of OTA and its metabolites and the occurrence of OTA in the faeces of animals dosed intravenously.

The binding of OTA to blood serum proteins determines its excretion rate and therefore its toxicity. Since in cattle as in humans and pigs binding is 2–3 times stronger than in sheep, the latter appear less prone to accumulation of OTA in blood (Blank et al., 2003). The high urinary pH of herbivores facilitates OTA clearance from blood compared to rhesus monkeys, pigs and rats (Blank et al., 2003). In general, chronic ingestion of comparatively low levels of OTA represents the situation in practice, and it can be considered the more critical scenario, having some potential to lead to accumulation in the long run.

OTα has been shown to be almost non-harmful on a cellular level in toxicology studies (Yamazaki et al., 1971; Creppy et al., 1983). The far weaker bondage of OTα to blood serum proteins and therefore faster excretion is generally considered to be another key feature for its much lower toxicity compared with OTA (Li et al., 1997; Chu, 1974).

4.3. Occurrence of OTA in ruminant tissues (meat)/products

For tissue of monogastric animals, there is clear evidence for the presence of OTA in pig meat and meat products (Gareis and Scheuer, 2000). Since OTA is accumulated in kidneys, this organ is contaminated most significantly, followed by liver, muscle and fat. The incidence of OTA in different pig-based sausages ranged from 46.7 to 77.2%, with maximum OTA concentrations of 3.16 or 4.56 µg/kg blood or liver type sausages, respectively (Gareis and Scheuer, 2000). OTA was essentially absent in meat from poultry, but present in considerable amounts in blood (Mortensen et al., 1983) and high concentrations in kidneys (Krogh, 1987).

Ruminant organs or muscle meat are not considered to be contaminated by OTA (Gareis, 1996). Given the accumulation in the kidney in other species, this would be expected to be the most likely tissue to be OTA contaminated. Overall, OTA was only detected in five of 31 beef sausage samples. It was noted that OTA contamination of ruminant meat products may also arise from the use of pig blood plasma and OTA contaminated spices (Gareis and Scheuer, 2000).

In calves fed diets containing OTA, the non-toxic cleavage product OTα has been found in trace amounts in the blood and at levels of <10 µg/kg in kidneys (Patterson et al., 1981). When two cows were fed a diet containing OTA at 317 to 1125 µg/kg of feed for 11 weeks, OTA was found in some tissues but not in milk (Shreeve et al., 1979). In the later study, muscle contamination was investigated, but results not reported explicitly, indicating no presence of OTA in this tissue.

4.4. Occurrence and transfer of OTA into ruminant milk

In general, carry-over of OTA into ruminant milk is considered to be of little significance (Blank, 2002; Fink-Gremmels, 2008), especially if compared with monogastric species or other

mycotoxins like aflatoxin. However, some studies found a transfer of OTA into ruminant milk, too. OTα was found in the milk of cows when being fed an OTA contaminated diet (in a single dose of 13.3 mg/kg and daily doses of 1.66, 0.75 and 0.25 mg/kg; one cow per treatment) (Ribelin et al., 1978). For the cow with the very high single dose, OTA was found in milk in high amounts one day after the treatment. Traces of OTA were found on days 3, 4 and 5 after the start for the cow on the 1.66 mg/kg dose of OTA, and no OTA was found at daily doses <1.66 mg/kg. In goats given a single dose of 0.5 mg OTA/kg, recovery of OTA and OTA metabolites in milk was about 6% of the total excretion (faeces: ~54%; urine 38%; 2.3% in serum) (Nip and Chu, 1979). Of this 6%, about 14% were in the form of OTA, and the rest in the form of 3 other metabolites not further characterised.

In other experimental studies no transfer of ochratoxins into milk of ruminants could be shown: Concentrations of 0.317–1.125 mg OTA/kg in the concentrate (diet as offered: 10 kg concentrate + 9 kg hay per day) did not lead to detection of OTA or OTα in the milk of cows (Shreeve et al., 1979). In ewes being offered 1.5 kg hay plus 0.5 kg OTA-contaminated concentrate (OTA content in concentrate 0.04 mg/kg, approximate dose 0.2 µg/kg bw), no ochratoxin was found in milk (Jolánkai et al., 2007) after a period of 10 days, too, just as in another study by these authors using regular sheep diets only occasionally contaminated with OTA (Jolánkai et al., 2008).

In surveys on samples from milk producers, no OTA and no or only traces of OTα were detected in 121 samples of cow milk from northern Germany (Valenta and Goll, 1996). Another study also found no OTA in 69 milk samples from Germany (Engel, 2000). OTA was also absent in 100 dairy milk samples of a milk survey in the UK (Food Standard Agency, 2001). In this study, 100 milk samples (50 retail and 50 farm gate), both produced conventionally and organically, were analysed. Spanish studies also found no OTA in 48 (González-Osnaya et al., 2008) and 12 (Bascarán et al., 2007) milk samples.

On the contrary, traces of OTA were found in cow milk in a Swedish study (Breitholtz-Emanuelsson et al., 1993). A total of 36 cow milk samples were analysed. OTA was found in five (14%) of the samples (range 0.01–0.04 ng/mL). Small traces of OTA in milk were also found in a Norwegian study (Skaug, 1999). Samples of cow milk from organic and conventional farms and cow milk-based infant formulas were analysed. OTA was detected in six out of 40 cow milk samples from conventional farms (range 0.011–0.058 ng/mL) and in five out of 47 organic farm milk samples (range 0.015–0.028 ng/mL). OTA was not detected in any of the 20 infant formula samples. The OTA levels in cow milk found in this investigation were sufficient to cause a higher intake of OTA than the suggested tolerable daily intake of 5 ng/kg bw, e.g. in

small children who consume large quantities of milk. In a survey on raw bulk milk in the northwest of France, in 132 farms surveyed in winter and summer using diets based on maize silage and containing large portion of farm produced cereal grains, the overall incidence of milk contamination with OTA at farm level was low (Boudra et al., 2007). OTA was detected in three out of 264 samples at a low level of 0.005 to 0.008 ng/mL. OTα was not determined in this study, so it remains unclear whether the low occurrence of OTA was due to a very effective degradation of OTA in the rumen, or due to low contamination of the feeds used by the farmers. A study on five farms in the Sudan detected OTA in cattle milk in one sample at a very high concentration of 2.73 ng/mL. The animals were fed a ration containing OTA at a level of approximately 0.61 µg/kg ration (Elzupir et al., 2009). In a survey on dairy products (n = 195) in Germany, low OTA incidence and concentrations were found; the most prominent contamination was found for cheese with ingredients, indicating that like in meet products, spice ingredients contribute more to OTA contamination than the animal product itself (Engel, 2000).
If present at all, OTA content of milk ranged from 0.005–0.058 ng/mL in different studies.
Although this concentration in the milk is not to be regarded as a severe and drastic problem for the consumers it shows that limited transfer of OTA into milk can occur under certain circumstances. It needs to be added that a direct animal-independent OTA contamination of milk after the milking process can not be excluded completely.

5. Concluding Remarks and Considerations

Traditionally, OTA has been regarded as relatively uncritical for ruminants. Based on *in vitro* trials, 12 mg OTA/kg feed were estimated to be degradable by ruminants (Hult, 1976). Another author describes repeated doses of 33–72 mg OTA/d for cattle and 3–7 mg OTA/d for sheep as being degradable by the animals (Müller, 1995). This would correspond to amounts of approximately 200 µg/kg bw (Hult, 1976) (assuming 1.2 kg daily feed intake and 70 kg sheep) or 40–120 µg/kg bw (Müller, 1995) (assuming 450–600 kg cattle and 45–70 kg sheep). In fact, at estimated doses of 40–100 µg/kg bw (sheep; assuming 60 kg bw and1.2 kg food intake) (Kiessling et al., 1984) could not detect any OTA in blood serum. However, it needs to be added here that in older studies, analytical limits for OTA of 75 ng/mL (Kiessling et al., 1984) are almost two orders of magnitude above today's levels of app. 1 ng/mL (Blank, 2003). In fact, based on improved and more sophisticated analytical methodology and equipment, some more recent evidence is available that the effect of OTA on ruminants in this respect is generally underestimated. At concentrations described to be safe, OTA occurs systemically in significant amounts (Höhler et al., 1999; Blank, 2003; Blank and Wolffram, 2009). Therefore, although no

obvious pathological effects occur, the common view that OTA is degraded completely by an active rumen microbial population under all circumstances does not hold true.

The most likely explanation is that rumen bypass of intact; undegraded OTA needs to be taken into account. Even when assuming an active microbial population the working modus of the ruminal forestomach always leads to some bypass of feed components, the more comprehensive the more these substances resemble fluids in their mixing and passage behaviour from the rumen. Following an approach to estimate ruminal protein degradation (Ørskov and McDonald, 1979; McDonald, 1981), assuming a best case (degradation rate in the rumen: 0.40 h^{-1}; passage rate from the rumen 0.02 h^{-1}) or a worst case (degradation rate in the rumen: 0.06 h^{-1}; passage rate from the rumen 0.10 h^{-1}) scenario results at estimates of OTA bypass of 5 up to 62 % of the dose, explaining the systemic occurrence of undegraded OTA in significant amounts irrespective of an intact and active rumen microbial population experienced by some of the more recent studies. Other authors arrive at estimates of a maximal rumen bypass of intact OTA of 10% (Müller, 1995).

Suitable feed additives are a future perspective to further reduce any risk of OTA in ruminants. The pH-stabilizing effects of yeasts and the potentially beneficial effect on the microbial population have already been investigated in this respect, however did not influence OTA degradation and systemic availability in the study of (Blank and Wolffram, 2009).

While several studies on OTA in ruminants have improved our knowledge significantly over the last three decades, some knowledge gaps appeared to us in the process of summing up this review. From the point of view of basic research, further systematic *in vitro* trials on the influence of diet and the relevance of different microbes on ruminal degradation of OTA appear desirable to further clarify the role of individual groups. Critical regarding the practical relevance of OTA in ruminants would be information of systemic occurrence levels and transfer into milk in ruminants fed at the high feeding levels typical for lactating animals for sufficiently long period (1–3 months) at praxis-relevant OTA-concentrations (on the lower range of those reported in Table 7). This will further assure judgements of the potential of OTA to induce harmful effects in ruminants.

6. References and Notes

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4 Ruminal ochratoxin A degradation – contribution of different microbial populations and influence of diet

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Abstract

The mycotoxin ochratoxin A (OTA) is degraded extensively in the rumen. In this study, the relative contribution of different rumen microbial populations (MP) and the effect of diet on degradation of OTA were evaluated in a factorial design experiment. Degradation of OTA was quantified by using the Hohenheim gas test (HGT) *in vitro* fermentation system. Five different HGT diets were used (concentrate:forage proportions (C:F) – 10:90, 30:70, 50:50, 70:30, 90:10), and donor animals were fed diets with the respective ratio. Diets with the highest concentrate content were supplied with and without 10 g/kg sodium bicarbonate (70:30 BC and 90:10 BC). The MP included whole rumen fluid, fungi+protozoa, bacteria+protozoa, protozoa and bacteria+fungi. Protozoa numbers were counted after 24 h and OTA and ochratoxin alpha (OTα) analysed at 0, 4, 8, 12, 24 h. Area under the curve (AUC) and half-life were calculated for the latter two. The short average OTA half-life for whole rumen fluid of 2.6 h (1.3 to 4.5 h) demonstrates the high OTA degradation capacity of the rumen MP (*i.e.*, standard HGT inoculum) and corresponds well with published *in vivo* results. Both MP and diet affected OTA degradation. Interactions among factors occurred (P<0.001), which made it necessary to do further comparisons within factor levels. Among MP, those with bacteria (bacteria+fungi and bacteria+protozoa) had lower AUC values (P<0.001) for OTA (196 to 673 ng/ml × h, meaning higher degradation capacity, than those without bacteria (fungi+protozoa and protozoa; 701 to 1206 ng/ml × h). Whole rumen fluid had the lowest AUC values (146 to 249 ng/ml × h; P<0.05). Diet had a quadratic effect (P=0.001) on protozoal numbers with minimum values for the lowest and highest C:F ratios, for bacteria+protozoa, fungi+protozoa and protozoa, but no corresponding effect was found for OTA degradation parameters. Whilst the generally high capacity to degrade OTA was confirmed, results for the contribution of different microbial groups shed new light on ruminal OTA degradation.

1. Introduction

Mycotoxins are a large diverse group of naturally occurring secondary metabolites of fungi. Among ochratoxins, ochratoxin A (OTA) is the most prevalent metabolite of some toxigenic species of *Aspergillus* and *Penicillium* genera (Chu, 1974; Frisvad and Samson, 1991). *Penicillium* tends to be more prevalent in cooler climates whereas in warmer climates *Aspergillus* is of more relevant (Krogh, 1987). Ochratoxin A has been found as a natural contaminant in food and feedstuffs (Shotwell et al., 1969; Dwivedi and Burns, 1986). Contamination levels of feedstuffs can be higher than 100 µg/kg (Kuiper-Goodmann and Scott, 1989), but most feeds which test positive will be below this level. While concentrates can be contaminated with OTA, forages are generally not considered as an important source of OTA (Mobashar et al., 2010). Therefore, the potential for OTA contamination of ruminant diets increases with the concentrate proportion of the diet.

Ochratoxin A is a complex compound containing ochratoxin α (OTα) which is linked via a 7-carboxy group to L-ß-phenylalanine by an amide bond. Exposure to OTA causes a variety of pathological responses such as nephrotoxic, hepatotoxic, teratogenic and carcinogenic in non-ruminants, with nephropathy commonly occurring in swine (Krogh, 1987; Marquardt and Frohlich, 1992). However, ruminants are relatively resistant to its toxic effects due to detoxification of OTA to its less toxic metabolite, OTα and phenylalanine by rumen microbes (Ribelin et al., 1978; Kuiper-Goodmann and Scott, 1989; Blank et al., 2004; Driehuis et al., 2010; see Mobashar et al. (2010) for a recent review on OTA in ruminants). Several *in vitro* studies using ruminal fluid collected from cows and sheep, and administration of OTA into the rumen of calves, sheep and goats, have reported OTA hydrolytic capacity of rumen microbes (Hult et al., 1976; Nip and Chu, 1979; Müller et al., 1998). Based on *in vitro* estimations, ruminants are able to degrade up to 12 mg of OTA/kg contaminated feed (Hult et al., 1976; Kiessling et al., 1984). While such *in vitro* studies mostly do not consider passage of digesta out of rumen and the following absorption in the lower gastrointestinal tract (rumen escape), they have considerable merit in evaluating principles of ruminal degradation.

Former studies have indicated that 0.88 to 0.90 of total hydrolysis of OTA to less toxic metabolites is associated with rumen protozoa and only 0.10 to 0.12 with bacteria (Galtier and Alvinerie, 1976; Kiessling et al., 1984; Xiao et al., 1991). According to previous studies degradation and systemic availability of OTA depends on concentrate:forage (C:F) ratio in the diet, which in turn seems to be important to maintain composition of an active rumen microbial population (MP) with respect to OTA degradation.

This study aimed to evaluate effects of different diet types (C:F) and the contribution of different microbial groups (i.e., bacteria, protozoa and fungi) in the rumen to OTA degradation. Given the general emphasis on the role of protozoa regarding OTA degradation, their role was of particular interest.

2. Materials and methods

2.1. Donor animals and feeding regimes

Rumen fluid was obtained from three fistulated Blackface ewes (60±10.0 kg body weight). Diets fed to the animals were (C:F ratio, dry matter (DM) basis) 10:90, 30:70, 50:50, 70:30 BC (sodium bicarbonate), 70:30, 90:10 BC and 90:10. Bicarbonate was added at 10 g/kg of the diet. The forage was chopped grass hay and the concentrate (g/kg) was 261 solvent-extracted rapeseed meal, 180 palm kernel expeller, 180 wheat middlings, 150 wheat gluten feed, 141 soybean hulls, 70 beet vinasse, 16 $CaCO_3$ and 2 NaCl. The diet was given in two meals at 07:00 and 15:30 h. Ewes were fed each diet for a 10 to 14 d adaptation period. Water was available *ad libitum*. The chemical composition of donor animal diets calculated from the means of concentrate and hay samples of all periods is in Table 9.

The average chemical composition (± SD; g/kg DM) of concentrate over all periods was: ash 86 ± 3.4, crude protein (CP) 240 ± 8.1, ether extract (EE) 42 ± 6.7, neutral detergent fibre (assayed with a heat-stable amylase and expressed exclusive of residual ash; aNDFom) 375 ± 3.4, acid detergent fibre (expressed exclusive of residual ash, ADFom) 192 ± 26.2 and lignin(sa) 69 ± 12.4, and for hay: ash 105 ± 5.9, CP 123 ± 8.6, EE 27 ± 6.2, aNDFom 602 ± 21.6, ADFom 307 ± 28.0 and lignin (sa) 59 ± 6.7.

Table 9: Dry matter (DM) content (g/kg) and composition[a] (g/kg DM) of donor animal diets

Diet (C:F[b])	DM	Ash	CP	EE	aNDFom	ADFom	Lignin (sa)	NFC[c]
	g/kg	g/kg DM						
10:90	909	103	135	28.3	579	296	65.4	155
30:70	906	99.6	158	31.5	534	273	61.9	177
50:50	903	95.8	181	34.6	488	250	63.9	201
70:30 BC[d]	890	91.1	203	37.3	438	224	65.3	231
70:30	899	92.1	205	37.7	443	227	66.0	222
90:10 BC	887	87.4	226	40.4	393	202	67.3	253
90:10	896	88.3	228	40.8	397	204	68.0	246

[a]CP, crude protein; EE, ether extract; aNDFom, neutral-detergent fibre assayed with a heat-stable amylase and expressed exclusive residual ash; ADFom, acid-detergent fibre expressed

exclusive residual ash; lignin (sa), lignin determined by solubilization of cellulose with sulphuric acid; [b]Concentrate:forage; [c]NFC = non-fibre carbohydrate (1000 - aNDFom - CP – EE - Ash); [d]Sodium bicarbonate.

Table 10: Dry matter (DM) content (g/kg) and composition[a] (g/kg DM) of Hohenheim gas test (HGT) diets (as calculated from concentrate and forage components)

Diet (C:F[b])	DM	Ash	CP	EE	aNDFom	ADFom	Lignin (sa)	NFC	OTA
	g/kg				g/kg DM				µg/kg DM
10:90	897	103	117	26.8	540	307	45.4	214	18.8
30:70	891	83.7	121	25.6	444	246	37.6	325	18.3
50:50	887	64.8	126	24.5	348	184	29.9	437	18.7
70:30	881	45.9	130	23.3	252	123	22.1	549	19.0
90:10	875	27.0	134	22.1	156	61.4	14.3	661	19.6

[a]CP, crude protein; EE, ether extract; aNDFom, neutral-detergent fibre assayed with a heat-stable amylase and expressed exclusive residual ash; ADFom, acid-detergent fibre expressed exclusive residual ash; lignin (sa), lignin determined by solubilization of cellulose with sulphuric acid; NFC, non-fibre carbohydrate (1000 - aNDFom - CP – EE - Ash); [b]Concentrate:forage

2.2. Samples for Hohenheim gas test

The Hohenheim gas test (HGT) diets had C:F of 10:90, 30:70, 50:50, 70:30 and 90:10 and were prepared by mixing different levels of wheat grain and grass hay. In the incubations with the inoculum from sheep fed with additional rumen buffer (sodium bicarbonate), no additional buffer was added to the *in vitro* incubations since the amounts of bicarbonate used in the HGT were considered sufficient to keep pH stable for any feedstuff used. The diets were contaminated with OTA-contaminated wheat grain produced by inoculation of wheat with *Aspergillus ochraceus* NRRL 3174. The average OTA content was 18.6 µg OTA/g DM (Table 10 lists values of the individual diets), which is comparable to the highest levels reported in the feeds. Samples were milled by using a 1 mm sieve in a centrifugal mill (type ZM1; Retsch, Haan, Germany) and stored in airtight plastic containers. The chemical composition of HGT diets is in Table 10.

Chemical analysis was done according to the German Handbook of Agricultural Experimental and Analytical Methods (VDLUFA, 2007) and method numbers are given. Ash and ether extract were analysed using methods 8.1 and 5.1.1 respectively. Crude protein was determined by Dumas combustion (4.1.2). Neutral detergent fibre (6.5.1; assayed with heat

stable amylase) and acid detergent fibre (6.5.2) are expressed exclusive of residual ash and therefore designated aNDFom and ADFom, respectively. Lignin (sa) was determined by solubilization of cellulose with sulphuric acid.

2.3. Rumen fluid collection and microbial treatments

The rumen fluid collected from fistulated ewes before morning feeding was strained and squeezed through double layered cheesecloth. A continuous flow of CO_2 was supplied to maintain anaerobic conditions. This rumen fluid was the basis for the rumen microbial population treatments, namely whole rumen fluid (WRF; standard HGT inoculum, as a control), fungi+protozoa (F+P), bacteria+protozoa (B+P), protozoa (P) and bacteria+fungi (B+F). According to each treatment, a particular rumen microbial group was switched off and/or depressed from the rumen fluid. Bacteria and fungi were eliminated chemically by using antibiotic (Penicillin-G plus streptomycin sulphate; Dehority and Tirabasso, 2000) and fungicide solutions (Clotrimazole) respectively. Protozoa were removed from rumen fluid by standard centrifugation procedure (Mendoza et al., 1995), and the supernatant was used as the inoculum. Some overview on application of rumen microbial population treatments is in Table 11.

Table 11: Procedure for the establishment of particular microbial populations (MP) in the rumen fluid

Whole rumen fluid	No MP depression	Standard Hohenheim gas test (HGT) procedure	Menke et al. (1979)
Fungi+protozoa	Chemical depression of bacteria	Standard HGT conditions, except: overnight pre-incubation of rumen liquor in Woulff bottle under continuous supply of CO_2, with freshly prepared antibiotic solution (60000 U penicillin-G (Sigma, St Louis, MO) 20.5 µg/ml inoculum + 3900 U streptomycin sulphate (U.S. Biochemical Corp Cleveland, Ohio) 2.91 µg/ml inoculum, dissolved in millipore water). Antibiotic dose was 1 ml of antibiotic solution per 30 ml rumen inoculum. Early in the morning, the same dose of antibiotic solution was supplied again to the rumen inoculum and mixed about 1h.	On basis of Dehority and Tirabasso (2000)
Bacteria+protozoa	Chemical depression of fungi	As for fungi+protozoa, except the replacement of antibiotics by freshly prepared fungicide solution (clotrimazole (US, Biochemical Corp) 0.49 µg/ml inoculum dissolved in millipore water). Fungicide dose was 1 ml of fungicide solution per 30 ml rumen inoculum.	
Protozoa	Chemical depression of bacteria and fungi	As the fungi+protozoa and bacteria+protozoa, except that antibiotics and fungicide were applied simultaneously.	
Bacteria+fungi	Elimination of protozoa by physical procedure	Standard HGT conditions, except that supernatant after centrifugation of fresh rumen fluid at 200 g for 3 min was used as inoculum source	Mendoza et al. (1995)

Table 12: Protozoa counts of the different diets and rumen microbial populations (MP; 24 h incubations); each value in column is a least square mean of 4 replicates

		Protozoa count ($\times 10^6$/ml)
Ration (C:F[a])	10:90	7.1
	30:70	13.2
	50:50	16.3
	70:30 BC[b]	18.7
	70:30	17.6
	90:10 BC	17.5
	90:10	16.6
MP	Whole rumen fluid	20.1
	Fungi+protozoa	18.3
	Bacteria+protozoa	18.4
	Protozoa	18.4
	Bacteria+fungi	1.2
SEM		0.71
Significance (P-value)		
Diet		<0.001
MP		<0.001
Diet × MP		<0.001

[a]Concentrate:forage; [b]Sodium bicarbonate.

Table 13: Results of comparisons of means (contrasts) within factor level for protozoa counts of the different diets and microbial populations (24 h incubations)

	Contrast (*P*)			
Diet (C:F[b])	WRF *vs* F+P, B+P and P	F+P, B+P and P *vs* B+F	F+P and B+P *vs* P	F+P *vs* B+P
10:90	<0.001	<0.001	0.024	<0.001
30:70	0.144	<0.001	0.595	0.430
50:50	<0.001	<0.001	0.621	0.237
70:30 BC[c]	0.002	<0.001	<0.001	0.046
70:30	0.527	<0.001	0.761	1.000
90:10 BC	<0.001	<0.001	<0.001	0.018
90:10	0.005	<0.001	0.382	0.742

MP	Linear	Quadratic	Cubic	BC *vs* no BC[d]	70 *vs* 90	70+90 BC *vs* 70 BC+90
WRF	0.093	0.742	0.697	0.015	0.004	0.002
F+P	<0.001	<0.001	0.105	0.049	<0.001	<0.001
B+P	<0.001	<0.001	0.411	0.014	<0.001	0.014
P	<0.001	<0.001	0.902	0.050	0.658	<0.001
B+F	0.939	0.742	0.697	0.834	0.658	0.592

[a]Whole rumen fluid (WRF), fungi+protozoa (F+P), bacteria+protozoa (B+P), protozoa (P), bacteria+fungi (B+F); [b]Concentrate:forage; [c]Sodium bicarbonate; [d]BC vs no BC = 70:30 + 90:10 vs 70:30 BC + 90:10 BC; 70 vs 90 = 70:30 + 70:30 BC vs 90:10 + 90:10 BC; 70+90 BC vs 70 BC + 90 = 70:30 + 90:10 BC vs 70:30 BC + 90:10.

To confirm protozoal elimination, they were enumerated in all microbial treatments at 24 h, using 1 ml of incubated sample per 9 ml of 40 ml/l formaldehyde solution. Total protozoal counts were made in 30 microscopic fields at a magnification of 100x using a Fuchs-Rosenthal counting chamber (depth 0.2 mm, 0.0125 µl/grid). Both diet and MP had a significant effect on protozoal numbers, with interactions between the factors (Table12). The major goal was achieved and protozoa were successfully reduced by one order of magnitude in the bacteria+fungi treatment. Except for WRF and the protozoa-depleted MP, linear and quadratic effects of diet on protozoal numbers occurred (Table 13, Figure 4), with maximum values at moderate dietary concentrate proportions.

2.4. In vitro incubation and sample collection

The incubation medium was prepared according to Steingass and Menke (1986). Rumen liquor collected from donor animals was preincubated, in the case of fungi+protozoa, bacteria+protozoa and bacteria+fungi, with freshly prepared antibiotic and fungicide solutions overnight with a continuous supply of CO_2 at the rate of 1 l/min. Early in the morning antibiotic and fungicide solutions were again supplied to the rumen inoculum and mixed for about 1 h to create the rumen inoculum treatment with double antibiotic and fungicide concentrations. Test diets samples (200 ± 2.0 mg) were weighed into 100 ml calibrated glass syringes fitted with plunger as described by Menke et al. (1979). The pH of freshly collected rumen fluid was measured and ranged from 6.6 to 6.9. All syringes along with three blanks were incubated in Hohenheim gas test incubator (Menke et al., 1979) for 24 h and gas production was recorded at 4, 8, 12 and 24 h. Contents of syringes were stored frozen until OTA and OTα analysis.

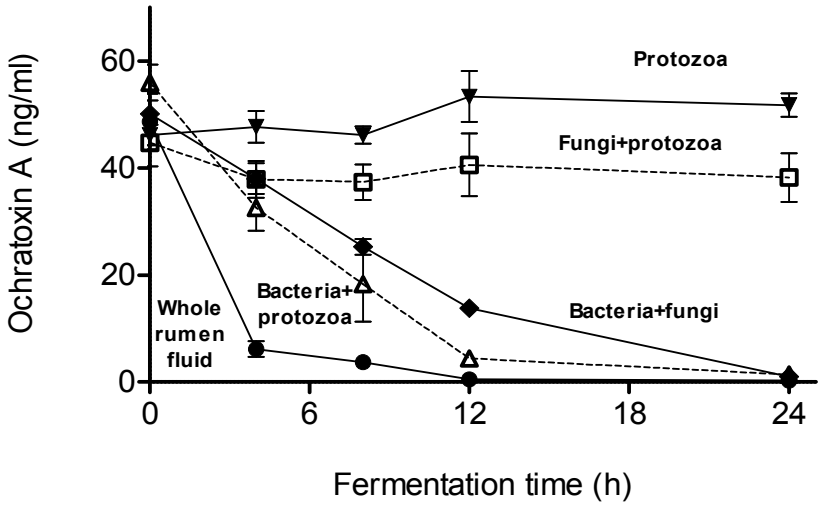

Fig. 2. Degradation of ochratoxin A by the different microbial populations in the HGT (Hohenheim gas test) with a concentrate:forage ratio of 50:50 (error bars represent SEM)

2.5. Extraction procedure for OTA

Extraction of OTA from the samples was according to Blank et al. (2003). Eight millilitres of acid inactivated sample (pH 2.1, acidified with H_3PO_4) were extracted for 20 min with 15 ml of chloroform under constant shaking, followed by centrifugation at 10,000 × *g* for 30 min at 2°C in 50 ml polyethylene tubes to separate chloroform and aqueous phases. The upper water layer was removed, and the chloroform fraction was transferred into a 30 ml centrifuge tube added with 2 ml of deionised water, and the mixture was shaken for 5 min, followed by centrifugation at 4,000 × g for 10 min at 2°C. A 5 ml aliquot of washed chloroform fraction was transferred into a 6 ml glass tube and evaporated. Dry samples were reconstituted in 3 ml of methanol, followed by centrifugation at 4,000 × *g* for 10 min at 2°C and used for high performance liquid chromatography (HPLC) analysis

2.6. High performance liquid chromatography analysis

The HPLC analysis for OTA and OTα followed the procedure of Xiao et al. (1996). The HPLC system included a Waters 717 plus autosampler, a Waters 600E multisolvent delivery system, a Waters 474 scanning fluorescence detector, a Waters in line degasser, and a Millipore/Waters TCM column oven system (Waters, Eschborn, Germany). An aliquot of 30 µl of the extracts dissolved in methanol was injected onto a 250 mm × 4.6 mm i.d. Nova-Pak_{18} column fitted with a 20 mm × 3.9 mm i.d., 4 µm Nova-Pak-C_{18} guard column. The

fluorescence detector was set at an excitation and emission wavelength of 330 and 450 nm, respectively. All HPLC analyses were at a solvent flow of 1.5 ml/min and an oven temperature of 40°C using one of two solvent gradient elution profiles consisting of deionised water acidified to a pH of 2.1 with H_3PO_4 (solvent A) and a mixture of methanol and 2-propanol (90/10 v/v, solvent B). To facilitate separation of OTA and OTα from interfering compounds, a short gradient was used. The short gradient was programmed to deliver 50 to 25% A from 0 to 12 min, 25 to 10% A from 12 to 12.1 min, 10% A from 12.1 to 17 min, 10 to 50% A from 17 to 17.1 min, and 50% A from 17.1 to 22 min. For preparations of standards, OTA and OTα were dissolved in pure ethanol and calibrated spectrometrically, based on their molar absorbance coefficient of 5500/M/cm at 333 nm for OTA and 6200/M/cm at 335 nm for OTα (Xiao et al., 1995). After calibration standards were evaporated, dried samples were reconstituted and diluted with methanol to concentrations of 1 to 100 ng/ml. Identification of OTA and OTα in samples was based on respective retention times of standards. All the samples were analysed in duplicate.

2.7. Parameters computations and statistical analysis

Ochratoxin metabolism was quantified via two statistical parameters: being half-life of OTA disappearance/OTα formation (h) and area under the curve (AUC) of OTA/OTα concentration (ng/ml x h). For calculation of half-life, the following model equations were used:

a) OTA disappearance: $Y = (Y_0 - \text{plateau}) \times e^{-k \times t} + \text{plateau} + \varepsilon$;

b) OTα formation: $Y = Y_0 + (\text{plateau} - Y_0) \times (1 - e^{-k \times t}) + \varepsilon$;

where: Y = OT concentration at time t, Y_0 = OT concentration at 0 h (ng/ml), k = rate constant (/h), plateau = constant (~ OT concentration at infinite times, ng/ml), and ε = residual error.

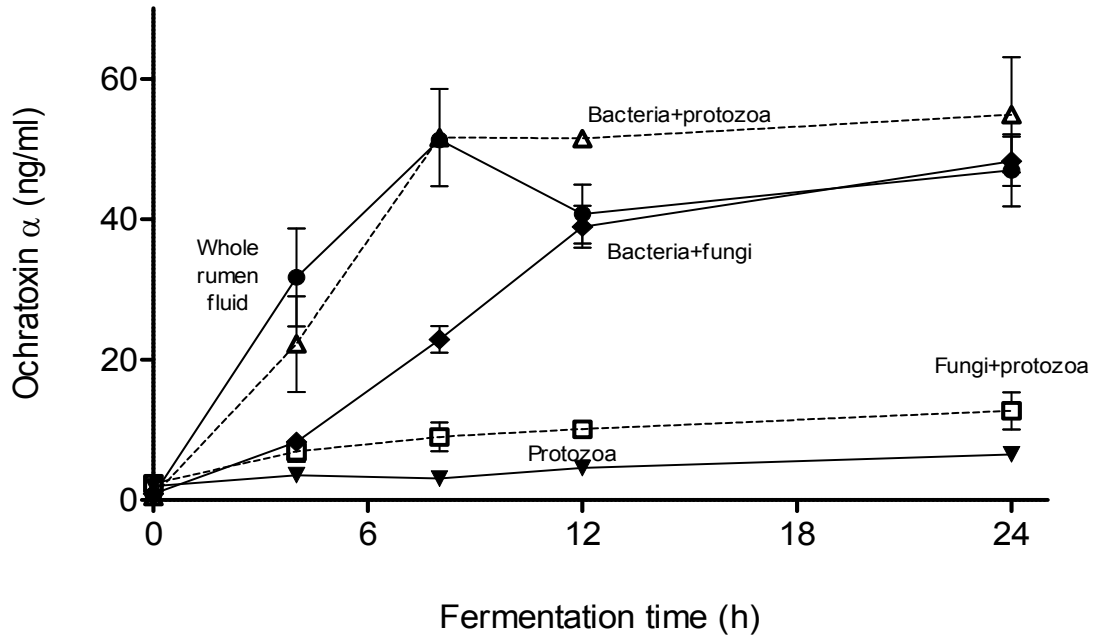

Fig. 3. Ochratoxin α formation by different microbial populations in the HGT (Hohenheim gas test) with a concentrate: forage ratio of 50:50 (error bars represent SEM)

Half-life was calculated as ln(2)/k. Calculation was only done for those MP which had considerable OTA degrading/OTα formation capacity (WRF, bacteria+fungi and bacteria+protozoa), which can be considered equivalent to a k significantly different from zero.

The AUC of OT concentrations was quantified by calculating the integral for the times 0 to 24 h. As such, it is regarded as a measure of bioavailability of a substance at a certain dosage (in contrast to half-life, which is independent of dosage). The term AUC is common in pharmacology, and has been used in studies on OTA in ruminants (Blank and Wolffram, 2009). The one phase decay/association (half-life of OTA/OTα) and the AUC procedures of Graph Pad Prism 5.0 (Graph Pad software, San Diego, CA, USA) were used for all calculations.

Analysis of the influence of factors on the dependant variables half-life and AUC (for OTA and OTα) and protozoal numbers was via the fully saturated statistical model including the main effects of diet, MP, and their interaction as:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + \varepsilon_{ijk,}$$

Where: Y_{ijk} = the observed response, μ = the population constant and is common to all observations, α_i = the effect of diet i; $i = 1 - 7$, β_j = the effect of MP j; $j = 1 - 5$, $(\alpha \times \beta)_{ij}$ = the effect of interaction between diet i and MP j and ε_{ijk} = the residual error.

For mean comparisons between diets, polynomial orthogonal (linear, quadratic and cubic) contrasts were completed for the five diets without BC. Linear orthogonal contrasts were to evaluate effects of BC in the diets of rumen fluid donor animals. Linear contrasts were also applied in mean comparisons of MP. In the case of a significant interaction, contrasts were done within factor levels (*i.e.*, individual diets or MP). The GLM and all further calculations used SAS (SAS, 2007). The standard level of significance for the effects was $P<0.05$. Effects with P values of $0.1>P>0.05$ are considered as trends. P values less than 0.001 are expressed as <0.001 rather than the actual value.

3. Results

The general pattern of OTA degradation and OTα formation by the microbial groups is visualized in Figures 2 and 3 for the diet with the intermediate concentrate proportion (C:F 50:50): Whilst the fastest degradation occurred with WRF, the populations bacteria+protozoa and bacteria+fungi both were able to degrade OTA to substantial degrees (although slower than WRF), while the populations fungi+protozoa and protozoa did not show important OTA degradation capacity even after 24 h of incubation (Figure 2). Formation of OTα occurred correspondingly, with considerable formation in WRF, bacteria+protozoa and bacteria+fungi, but little formation in fungi+protozoa and protozoa (Figure 3).

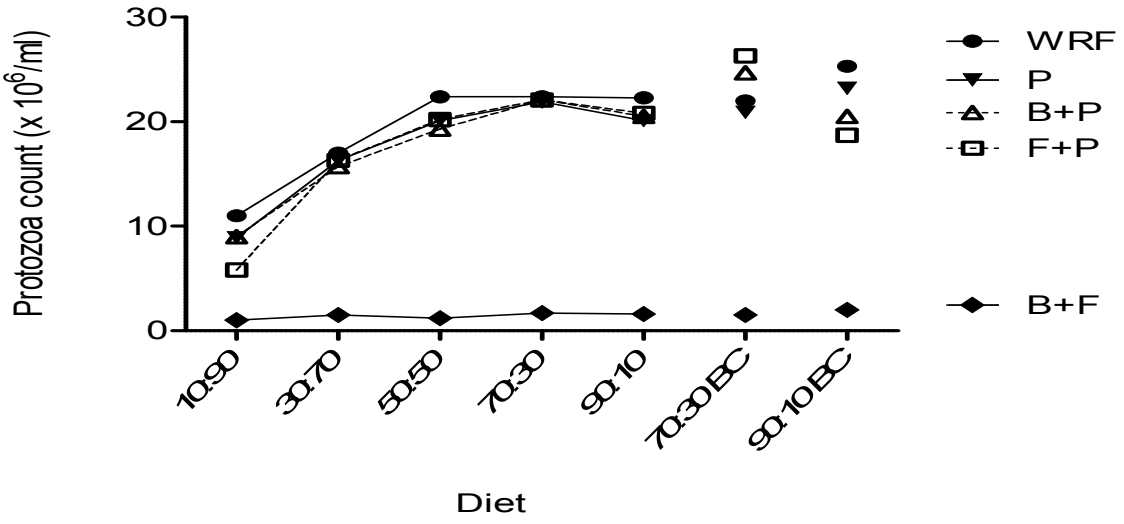

Fig. 4. Protozoal numbers for the different microbial populations and diets

Table 14: Half-life of ochratoxin A (OTA) and ochratoxin α (OTα) with different diets and microbial populations (MP; 24 h incubations; each value is a least squares mean of 4 replicates)

		Half-life (h)	
		OTA	OTα
Diet (C:F[a])	10:90	7.4	6.7
	30:70	5.4	5.6
	50:50	6.0	6.5
	70:30 BC[b]	5.5	7.9
	70:30	6.0	5.9
	90:10 BC	9.5	7.8
	90:10	12.9	9.0
MP	Whole rumen fluid	2.6	3.7
	Bacteria+protozoa	5.1	5.3
	Bacteria+fungi	14.8	12.3
SEM		0.90	0.66
Significance (P-value)			
Diet		<0.001	0.508
MP		<0.001	<0.001
Diet × MP		0.005	0.606
		Contrast (P-value)	
Diet	Linear		0.427
	Quadratic		0.392
	Cubic		0.707
	BC vs without BC[c]		0.779
	70±BC vs 90±BC		0.286
	70+90BC vs 70BC+90		0.779
MP	Whole rumen fluid vs with bacteria		<0.001
	Bacteria+protozoa vs bacteria+fungi		<0.001

[a]Concentrate:forage; [b]Sodiumbicarbonate; [c]BC vs no BC = 70:30 + 90:10 vs 70:30 BC + 90:10 BC; 70 vs 90 = 70:30 + 70:30 BC vs 90:10 + 90:10 BC; 70+90 BC vs 70 BC + 90 = 70:30+90:10BC vs 70:30BC+90:10.

3.1. Half-life of ochratoxin A and ochratoxin alpha

Half-life of disappearance of OTA ranged from 5.4 h to 12.9 h for the different diets and from 2.6 h to 14.8 h for the 3 MP, summarized as least squares means over all factor levels in Table 14. Both diet (P<0.001) and MP (P<0.001) had an effect on half-life of OTA, with interactions among factors (P=0.005). In the comparison of means within factorial levels (Table 15, Figure 5), half-life had a tendency to be shorter in WRF compared to the other two MP in 6 diets (P<0.1 at least), and half-life was shorter in bacteria+protozoa compared to bacteria+fungi in 4 diets (P<0.05). For WRF and bacteria+fungi, a quadratic effect of diet (*i.e.*, shorter half-life at intermediate concentrate levels) occurred (P<0.001), with the shortest half-life for the 50:50 diet with WRF and for the 70:30 diet with bacteria+fungi.

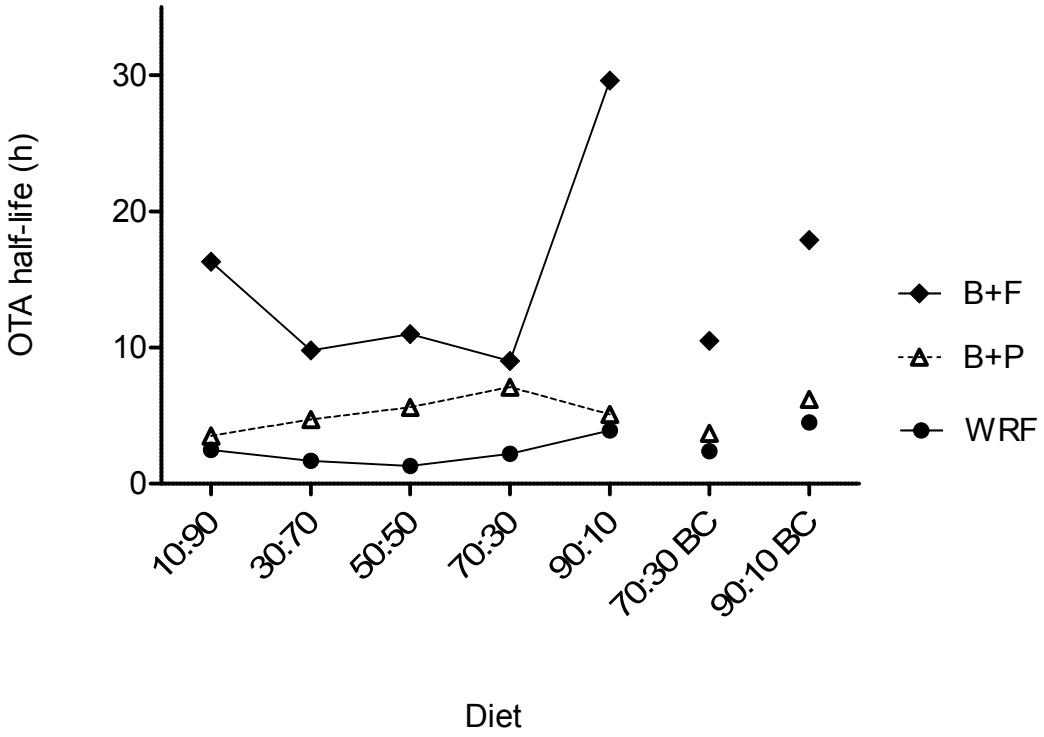

Fig.5. Half-life of OTA for different microbial populations and diets

Table 15: Results of comparisons of means (contrasts) within factor level for half-life of ochratoxin A (OTA) with different diets and microbial populations (24 h incubations)

	Contrast (*P*)	
Diet (C:F[b])	WRF *vs* with bacteria	B+P *vs* B+F
10:90	0.014	<0.001
30:70	0.058	0.133
50:50	0.018	0.113
70:30 BC[c]	0.112	0.048
70:30	0.055	0.611
90:10 BC	0.018	0.006
90:10	<0.001	<0.001

MP	Linear	Quadratic	Cubic	BC *vs* no BC[d]	70 *vs* 90	70+90 BC *vs* 70 BC+90
WRF	0.015	<0.001	0.818	0.870	0.431	0.943
B+P	0.354	0.378	0.556	0.711	0.941	0.444
B+F	0.038	<0.001	0.207	0.034	<0.001	0.007

[a]WRF whole rumen fluid, B+P bacteria+protozoa, B+F bacteria+fungi; [b]Concentrate:forage; [c]Sodium bicarbonate; [d]BC vs no BC = 70:30 + 90:10 vs 70:30 BC + 90:10 BC; 70 vs 90 = 70:30 + 70:30 BC vs 90:10 + 90:10 BC; 70+90 BC vs 70 BC + 90 = 70:30 + 90:10 BC vs 70:30 BC + 90:10.

Half-life for OTα formation, summarized as least squares means over all factor levels, ranged from 5.6 h to 9.0 h for the diets, and from 3.7 h to 12.3 h for the different MP (Table 14). The MP had an effect on half-life of OTα formation (P<0.001), while diet did not. No interaction between MP and diet occurred. In post-hoc tests summarizing all factor levels (*i.e.*, no diet x MP interaction), OTα formation was higher in WRF compared to the other populations (P<0.001), and in bacteria+protozoa compared to bacteria+fungi (P=0.001; Table 14)

3.2. Area under the curve for ochratoxin A and ochratoxin αlpha

For OTA, both diet (P<0.001) and MP (P<0.001) had an effect on AUC of OTA and an interaction between factors occurred (P<0.001; Table 16). The AUC values ranged from 508 to 668 ng/ml × h for the diets and from 199 to 1009 ng/ml × h for the different MP. In the post-hoc tests within factorial levels, WRF and other populations with bacteria (*i.e.*, bacteria+protozoa and bacteria+fungi) both had lower AUC values for all diets compared to populations without bacteria (fungi+protozoa; protozoa), and WRF had lower AUC than MP with bacteria (Table 17, Figure 6). In four of the diets, fungi+protozoa had a lower AUC than P.

Table 16: Area under the curve (AUC) of ochratoxin A (OTA) and ochratoxin α (OTα) with different diets and microbial populations (MP; 24 h incubations; each value in column is a least square mean of 4 replicates)

		AUC (ng/ml × h)	
		OTA	Otα
Diet (C:F[a])	10:90	563	405
	30:70	532	407
	50:50	625	386
	70:30 BC[b]	508	445
	70:30	580	362
	90:10 BC	668	310
	90:10	637	329
MP	Whole rumen fluid	199	630
	Fungi+protozoa	836	152
	Bacteria+protozoa	410	583
	Protozoa	1009	80.8
	Bacteria+fungi	483	443
SEM		28.2	20.9
Significance (P-value)			
Diet		<0.001	<0.001
MP		<0.001	<0.001
Diet × MP		<0.001	<0.001

[a]Concentrate:forage;[b]Sodiumbicarbonate.

Table 17: Area under the curve (AUC) of ochratoxin A (OTA) of different diets and rumen microbial populations (MP; each value is a least squares mean of 4 replicates)

Diet (C:F[b])	Contrast (*P*)					
	WRF *vs* without bacteria	WRF *vs* with bacteria	P *vs* F+P	With bacteria *vs* without bacteria		
10:90	<0.001	0.002	0.640	<0.001		
30:70	<0.001	0.032	0.002	<0.001		
50:50	<0.001	<0.001	0.004	<0.001		
70:30 BC[c]	<0.001	0.050	0.197	<0.001		
70:30	<0.001	<0.001	<0.001	<0.001		
90:10 BC	<0.001	<0.001	0.015	<0.001		
90:10	<0.001	<0.001	0.328	<0.001		
SEM						
	Linear	Quadratic	Cubic	BC *vs* no BC[d]	70 *vs* 90	70+90BC *vs* 70BC+90
WRF	0.063	0.528	0.185	0.347	0.537	0.508
F+P	0.407	0.166	0.188	0.668	0.006	0.019
B+P	0.004	0.843	0.576	0.072	<0.001	<0.001
P	0.698	0.002	0.392	0.175	0.619	0.129
B+F	0.205	0.089	0.120	0.540	0.548	0.470

[a]Whole rumen fluid (WRF), fungi+protozoa (F+P), bacteria+protozoa (B+P), protozoa (P), bacteria+fungi (B+F); [b]Concentrate:forage; [c]Sodium bicarbonate; [d]BC vs no BC = 70:30 + 90:10 vs 70:30 BC + 90:10 BC; 70 vs 90 = 70:30 + 70:30 BC vs 90:10 + 90:10 BC; 70+90 BC vs 70 BC + 90 = 70:30 + 90:10 BC vs 70:30 BC + 90:10.

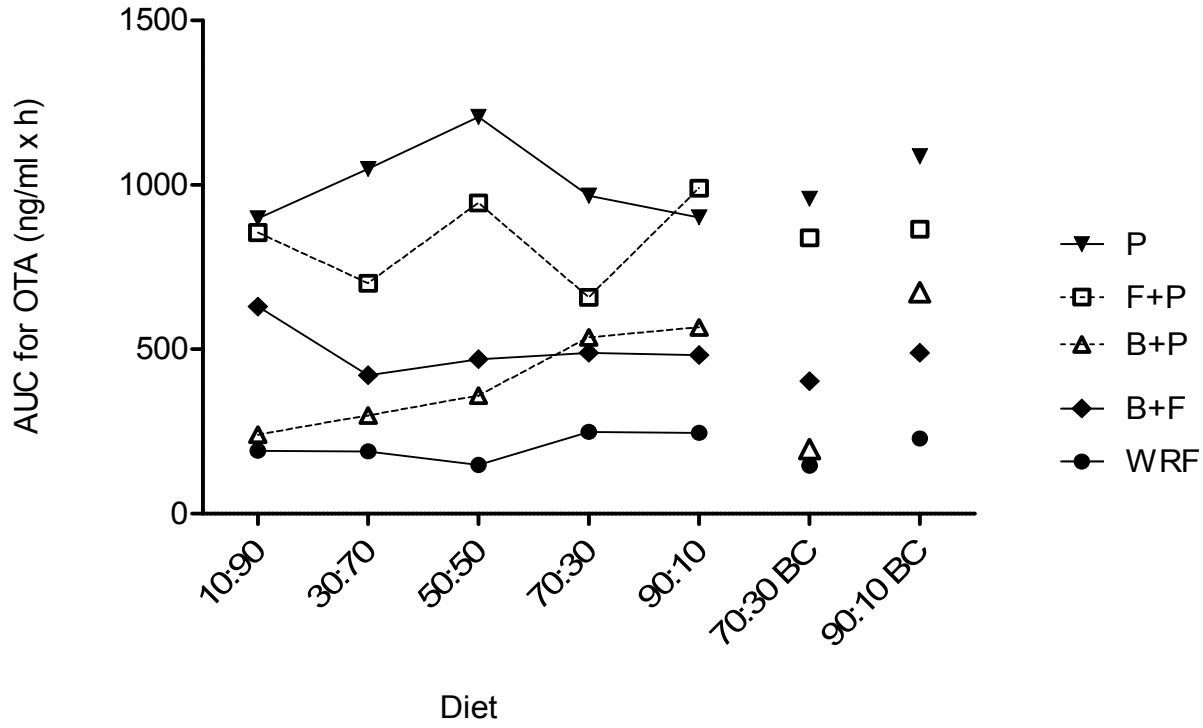

Fig.6. Area under the curve (AUC) of OTA for the different microbial populations and diets

For AUC of OTα, there was an effect of diet (P<0.001) and MP (P<0.001), with interactions among factors (P<0.001; Table 16). The AUC values ranged from 310 to 445 ng/ml × h for the diets and between 80.8 and 630 ng/ml × h for the different MP. In the post-hoc tests within factor levels, MP with bacteria (*i.e.*, WRF, bacteria+protozoa, bacteria+fungi) had higher AUC for OTα than those depleted of bacteria (*i.e.*, fungi+protozoa and protozoa; Table 18). In four of the diets, AUC of OTα was higher for WRF compared to bacteria+protozoa and bacteria+fungi.

4. Discussion

4.1. General aspects

It has long been recognised that functional ruminants are far less responsive to OTA toxicity than non-ruminants (Ribelin et al., 1978). This greater tolerance has been found to be based on ruminal degradation of the OTA molecule to far less toxic OT*α* and phenylalanine by the diverse MP in the forestomach (Galtier and Alvinerie, 1976; Hult et al., 1976). Of the general microbial groups occurring in the rumen (*i.e.*, bacteria, protozoa and fungi), and consistent

with their high proteolytic capacity (*i.e.*, detoxification of OTA = cleavage of an amide-bond), protozoa have been indicated by several studies to play the key role in OTA degradation (Galtier and Alvinerie, 1976; Kiessling et al., 1984; Xiao et al., 1991; Özpinar et al., 2002), and this opinion has been generally accepted.

Diet composition (*e.g.*, in terms of concentrate proportion) can have a major influence on OTA degradation, for example via its influence on the composition of MP. In line with the view of the role of protozoa, MP of animals fed extremely concentrate-rich diets (900 g/kg of concentrate, and therefore probably substantial protozoal depletion) had less OTA degrading capacity than those of sheep fed a diet higher in forage (Xiao et al., 1991).

Table 18: Area under the curve (AUC) of ochratoxin α (OTα) at different diets and rumen microbial populations (MP; each value is a least squares mean of 4 replicates)

	AUC (ng/ml × h) for OTα Microbial population[a]						Contrast (P-value)			
Diet (C:F[b])	WRF	F+P	B+P	P	B+F	*SEM*	WRF vs without bacteria	WRF vs with bacteria	P vs F+P	With bacteria vs without bacteria
10:90	636	203	728	84.6	374	60.0	0.001	0.100	0.047	0.001
30:70	697	166	621	63.4	488	58.1	0.001	0.006	0.084	0.001
50:50	596	143	659	67.5	462	56.0	0.001	0.495	0.204	0.001
70:30 BC[c]	633	188	766	92.6	547	60.6	0.001	0.650	0.109	0.001
70:30	692	121	457	77.0	464	55.8	0.001	0.001	0.458	0.001
90:10 BC	581	163	359	75.5	371	46.3	0.001	0.001	0.141	0.001
90:10	575	80.3	490	105	395	50.0	0.001	0.011	0.678	0.001
SEM	15.8	16.6	35.0	5.43	13.6					
	Contrast (P-value)									
Linear	0.194	0.079	0.008	0.298	0.807					
Quadratic	0.180	0.829	0.873	0.102	0.008					
Cubic	0.229	0.769	0.669	0.891	0.333					
BC vs no BC[d]	0.531	0.075	0.035	0.868	0.481					
70 vs 90	0.046	0.433	0.001	0.898	0.004					
70+90BC vs 70BC+90	0.441	0.852	0.001	0.591	0.204					

[a]Whole rumen fluid (WRF), fungi+protozoa (F+P), bacteria+protozoa (B+P), protozoa (P), bacteria+fungi (B+F); [b]Concentrate:forage; [c]Sodium bicarbonate; [d]BC vs no BC = 70:30 + 90:10 vs 70:30 BC + 90:10 BC; 70 vs 90 = 70:30 + 70:30 BC vs 90:10 + 90:10 BC; 70+90 BC vs 70 BC + 90 = 70:30 + 90:10 BC vs 70:30 BC + 90:

4.1.1. General methodological considerations

This study aimed at investigating the influence of diet and MP on OTA degradation. Thus, besides some variation in diet composition, provision of MP with substantial variability in metabolic activity was the first aim. This goal can be considered to have been achieved given the considerable differences in OTA degradation among groups. The chemical depression of parts of the MP apparently worked well. Also, the provision of a population depleted in protozoa was successfully performed and quantified by protozoal numbers of the treatments. The high protozoal numbers after 24 h can be taken as a guarantee of a comparable protozoal metabolic activity in the chemically depressed populations,of bacteria+protozoa, fungi+protozoa and protozoa. While quantification of the abundance of bacteria and fungi may have been desirable, it could not be completed in this study because of limitations of facilities and workload.

Two parameters integrating OTA degradation over the duration of 24 h incubation were used in this study. While a good inter-study comparability can be regarded as an advantage of the parameter “half-life” due to its independence of the OTA concentration used in the respective study, it can also be considered to be a major drawback because it is less applicable to populations showing little OTA degradation. Since the underlying model includes the calculation of a fractional OTA disappearance rate, this is not possible if the disappearance is low and some other variation occurs. For this reason, two MP (*i.e.*, fungi+protozoa and protozoa) were excluded from this analysis. In this respect, AUC is less demanding and is therefore a good parameter for comparison of OTA degradation capacity of all MP. However, AUC values cannot be compared easily among studies since they depend on the amount of OTA given.

Two parameters integrating OTA degradation over the total duration of 24 h incubation were used in this study. While a good inter study comparability can be regarded an advantage of the parameter “half-life” due to its independence of the OTA concentration used in the respective study; it can be considered a major drawback that it is less applicable to populations showing little OTA degradation. Since the underlying model includes the calculation of a fractional OTA disappearance rate, this is not possible if the disappearance is rather low and some random variation occurs. Due to this reason, we excluded two MP (protozoa+fungi and protozoa) from this analysis. AUC is less demanding in this respect, and therefore represents a good parameter for the comparison of OTA degradation capacity of all

five MP. However, AUC values cannot be compared easily between studies, since they depend on the amount of OTA actually given.

4.1.2. General capacity of rumen fluid for OTA degradation

The study demonstrated the ability of rumen fluid from sheep fed different diets to hydrolyse OTA and confirmed the high degradation capacity generally assumed for rumen contents. The average half-life of OTA for the WRF MP was 2.6 h (1.3 to 4.5 h for the different diets), compared to values of 5.3 h (2.0 to 12.7 h in Özpinar et al. (1999), 3.4 h (1.9 to 4.5 h in Özpinar et al. (2002) and 3.2 h (Blank et al., 2002), indicating an activity within the range of other *in vitro* studies. The general suitability of the HGT for investigations on OTA (such as the study of Blank et al., 2002) is also indicated by our study.

4.2. General results

In general, the effect of MP and diet on OTA degradation occurred in the GLM of all cases measured, which is a strong support for the view that these factors are important determinants of the degree of OTA degradation. In almost all cases, this was combined with interactions among factors, implying some change in the effect of a factor in relation to the level of the other factor.

4.2.1. Roles of rumen microbial groups

The influence of MP on OTA degradation was obvious and appeared more pronounced than that of diet. The general result can be visualised in Figure 1. The superior capacity of WRF, acting as a control, can be expected because it represents the most diverse and probably metabolically most active MP. A surprising result of the comparison of microbial groups is the clear indication for a major and dominant role of bacteria over protozoa in OTA degradation. Both populations incubated with antibiotics (penicillin and streptomycin sulphate, as used for bacterial depletion by Lee et al. (2000) showed a dramatic decrease of OTA degrading capacity compared to control. That even after 24 h, not all OTA was degraded in the bacteria-depleted (and not protozoa-depleted) populations show the limited effect of protozoa on OTA degradation. Given the slightly higher degradation of the bacteria+protozoa compared to the bacteria+fungi population across all diets (although not statistically significant), some contribution of protozoa exceeding that of fungi is suggested by the data, although it has to be regarded as considerably less to that of bacteria. While this should be

regarded as a major result of this study, our data should best be understood as a strong suggestion to rethink the role of bacteria and protozoa in ruminal OTA degradation, rather than the final proof for the roles of individual microbial groups.

While bacteria and protozoa contribute equally to total ruminal microbial mass in ruminants, it is well known that metabolic activity of bacteria far exceeds that of protozoa. Relative to protein degradation, the high degrading capacity of protozoa was often regarded as a good explanation for a high OTA degrading capacity. While this effect of protozoa is present, its source is mainly predation of bacteria by protozoa and not much protein degradation capacity *per se*. By feeding on bacteria, protozoa introduce an additional trophic level into the rumen ecosystem and therefore increase protein turnover rather than increasing the overall ruminal protein degrading capacity. Overviews on ruminal N metabolism (*e.g.*, Wallace et al., 1997) mention a particularly high dipeptidase activity as a characteristic of protozoa (Newbold et al., 1989; Wallace et al., 1996). However dipeptidase level has been found to be on the same level in defaunated animals, indicating no irreplaceable role of protozoa in this respect (Wallace et al., 1987). Bacteria are capable of all protein degrading cleavages, so a general metabolic limit appears unlikely. Indeed, at least some explicit indications of bacterial OTA degrading activity have already been provided for rumen bacteria by Schatzmayr et al. (2002), for a gastrointestinal tract MP free of protozoa (*i.e.*, rat caecum contents; Galtier and Alvinerie, 1976), and bacterial populations in various food processing/production processes (*e.g.*, Varga et al., 2000). Thus the general metabolic diversity and high metabolic activity of the bacterial populations make the outcome of our study more sensible. Retrospectively, the statement of Müller (1995; page 137/138) of no correlation of protozoal numbers to degradation rate of OTA may also be regarded as supporting the view of a less important role of protozoa in OTA degradation. In agreement with this statement, a quadratic relationship was found for dietary concentrate content and protozoal numbers with minimum values at the lowest and highest C:F ratios, while this was not the case for parameters describing OTA metabolism.

Our results contradict those of other investigations on the influence of rumen microbial groups, however the conditions of those studies (Galtier and Alvinerie, 1976; Kiessling et al., 1984; Xiao et al., 1991; Özpinar et al., 2002) differed from ours. It should also be mentioned that in the first study providing evidence for an overruling contribution of protozoa (Galtier and Alvinerie, 1976), the overall OTA degrading activity has to be considered very low (*i.e.*, only 0.50 after 24 h; in active populations: all is degraded after approximately 8 h) compared to most other studies.

The role of fungi in OTA degradation has been suggested to be potentially important (Müller, 1995), based on the high protease activity in some ruminal fungi (Wallace and Joblin, 1985), and the increased OTA degrading capacity of inoculum with favourable conditions for fungi. The effect of fungi alone seems to be limited (protozoa *versus* fungi+protozoa), although lower AUC indicating better degradation for fungi+protozoa occurred for 4 of 7 diets, withno difference in the 3 other diets).

The degrading capacity of the MP appeared to decrease with the number of microbial groups included, implying synergy between them. However, the presence of several groups does not appear necessary for hydrolysis of OTA, since OTA degradation includes only one step, and ther is no indication that the products inhibit the further reaction. However, OTA degradation should not be considered isolated from other reactions and some synergetic effect among microbial groups may occur for other metabolic reactions.

Work of establishment of different MP indicated a potential advantage of the HGT over some other *in vitro* systems in such investigations. In continuous systems such as RUSITEC it is well known that care has to be taken to keep protozoa in the population, but also in investigations using Woulff bottle incubations (Müller et al., 1998; Özpinar et al., 1999; Müller et al., 2001; Özpinar et al., 2002), protozoal numbers were decreasing by at least one order of magnitude within 24 h. This contrasts to our study where protozoal numbers were higher at 24 h compared to 0 h in all but one of all 35 MP/diet combinations, with an average increase of protozoal numbers of 20%. Given the long generation times of protozoa of 8 to 24 h (Van Soest, 1994) and generally high overall numbers found *in vitro* in our study compared to data from the rumen (summarized by Dehority, 2003; page 75) this can be interpreted as indicative of good conditions for protozoal growth.

4.2.2 Influence of diet

When considering the potential outcome of a variation of diet on OTA degradation, superior OTA degradation with diets of intermediate concentrate levels could have been expected; as such diets low in concentrate provide less energy and therefore support a less active MP, while those very high in concentrate may provide adverse growth conditions for many ruminal microbes. That quadratic effects for half-life of OTA in two populations occurred, implies a decrease of OTA degrading activity at the diet extremes of our study. However, no such effect occurred for AUC results, and therefore the presence of a decrease of OTA degradation at low and high concentrate proportions is equivocal for our data. Working in a well buffered *in vitro* system, the effect of a high concentrate proportion will likely have less

influence than in an animal, since the low rumen pH that will likely occur in an animal when fed a high concentrate diet will not occur *in vitro*. As our donor sheep were fed diets of the respective C:F ratio, some *in vivo* effect could be expected. However, the effect of high dietary concentrate proportions is considered to be far less for animals fed at maintenance intake level than for those at a high intake level. While our study gives some indication of a quadratic effect of protozoal numbers due to concentrate proportion with minimum values at the lowest and highest C:F ratios, such an effect did not occur for parameters describing OTA degradation.

5. Conclusions

With the methods applied here, a variety of microbial populations differing in their OTA degrading capacity were established. In contrast to the opinions in many publications, and previous predictions, the bacterial community played the dominant role in ruminal OTA degradation. There seems to be some OTA degrading capacity associated with protozoa, but it is much lower than that of bacteria. The evidence for an important role of fungi in OTA degradation is equivocal.

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5 Relative contribution of different rumen microbial co-cultures to gas production, short chain fatty acid and ammonium production from different diets in an *in vitro* fermentation system

Abstract

The primary objective was to elucidate the relative contribution of different rations and rumen microbial co-cultures to gas, short chain fatty acid (SCFA) and ammonium production at 24 h *in vitro* incubations using the Hohenheim gas test (HGT) procedure. Seven donor animal rations with different concentrate to roughage ratios (C:R: 10:90, 30:70, 50:50, 70:30, 70:30+$NaHCO_3$ (BC), 90:10 and 90:10BC), and five HGT rations (C:R: 10:90, 30:70, 50:50, 70:30 and 90:10) were formulated. The rumen microbial co-cultures included whole rumen fluid, fungi+protozoa, bacteria+protozoa, protozoa and bacteria+fungi and were established to achieve the goal of the present study. Physical and chemical methods were used to differentiate groups of bacteria, protozoa and fungi. Gas and ammonium production increased ($P<0.001$) as a result of a gradual increase in concentrate proportion of the rations. In general, SCFA production followed the same trend except the 90:10 and 90:10BC diets where SCFA production was lower. Supplementation of 70:30 and 90:10 rations with BC had no effect on SCFA production.

Whole rumen fluid and bacteria+fungi produced about 50% higher gas volume as compared to protozoa and fungi+protozoa fractions, whereas gas production with bacteria+protozoa was at intermediate level. Co-culture of protozoa either with bacteria or fungi produced more ammonium. Synergism among rumen microbes occurred in terms of SCFA production. In conclusion, variations in gas, SCFA and ammonium production from different rations and microbial co-culture treatments were attributed to relative proportion of concentrate and roughage in the ration and a particular microbial combination in rumen fluid.

1. Introduction

The rumen microbiota (bacteria, protozoa and fungi) can be considered as the key link between the ruminant animal and its diet. Each microbial species possesses a unique combination of characteristics inc1uding substrate utilized and type of fermentation products. The resulting fermentation products affect performance and efficiency with which feeds are utilized for production purpose (Baldwin, 1995). As a result relative proportions of nutrients available for absorption by the animal can be expected to change with diets, microbial populations and activity of individual rumen microbial species.

Protein metabolism

Often, bacteria have been reported to be primarily responsible for degradation of protein and subsequently releasing ammonia (Cotta and Hespell, 1986; Broderick et al., 1991; Wallace, 1996) while protozoa are thought to have a minor role (Chen and Wolin, 1979; Dennis et al., 1981; Nugent and Mangan, 1981), e.g. reported to be one-tenth of the specific activity of the bacteria (Forsberg et al., 1984). However other studies report synergism between bacteria and protozoa leading to higher ammonia production (Hino and Russell, 1987), or state that more than one-half of the proteolytic activity of mixed microbial populations is due to protozoa ((Warner, 1956; Blackburn and Hobson, 1960). Bacteria contribute 460 g/kg and protozoa contribute 200 g/kg to rumen nitrogen (Weller et al., 1958); however addition of protozoa to bacteria fermentations increases ammonium production as much as 40% (Luther et al., 1966).

Bacteria contribute to deamination of soluble protein while protozoa contribute to insoluble and particulate protein deamination (Hino and Russell, 1987). Thus physical form and changes in rumen microbial populations in the rumen are one of the most important factors in the relative contribution of bacteria and protozoa to the end products of rumen fermentation.

Carbohydrate metabolism

A complex consortium of rumen microbiota (bacteria, protozoa and anaerobic fungi) is considered to be responsible for degradation of polysaccharides of plant cell wall in the rumen (Schofield, 2000). Cellulolytic bacteria such as *Ruminococcus albus*, *R. flavefaciens* and *Fibrobacter succinogenes* are reported as chief microorganisms associated with ruminal digestion of plant cell wall ingested by the animal because of their numerical predominance and metabolic diversity (Stewart et al., 1981; Stewart and Bryant, 1988; Varel, 1989; Cheng et al., 1991; Dehority, 1993; Zhang et al., 2007). Nevertheless, protozoa and anaerobic fungi

have also been reported to be involved in fibre degradation. Depending on diet, protozoa have been demonstrated to digest 50-210 g/kg of the cellulosic materials (Dijkstra and Tamminga, 1995).

Moreover, Hungate (1955) suggested that one fifth of the fermentation acids produced in the rumen is contributed by rumen protozoa. Also Abou Akkada and EI-Shazly (1964) found a pronounced rise in SCFA levels (and ammonia) in faunated compared with protozoa free animals.

2. Materials and methods

All data on GP, SCFA and ammonium were quantified in the same trials as described in the previous chapter for the quantification of ochratoxin A (OTA) degradation.

2.1. Donor animals and feeding regime

Rumen fluid was obtained from three fistulated blackface ewes (60±10 kg body weight). The rations fed to the animals had concentrate:roughage ratios (C:R) of 10:90, 30:70, 50:50, 70:30 and 90:10; both rations with the highest concentrate proportions were also fed with $NaHCO_3$ (1% of the ration) (70:30BC; 90:10BC). The ration was given in two meals at 07:00 and 14:30 h. Animals were fed each ration for a 10 to 14 day adaptation period. Water was available ad libitum. The pH of freshly collected rumen fluid was measured and varied from 6.6 to 6.9 in the whole range of rations.

2.2. Samples for Hohenheim gas test (HGT)

Test rations for HGT were (C:R) 10:90, 30:70, 50:50, 70:30, 90:10 with different levels of wheat and grass hay. Samples were milled by using 1 mm sieve in a Retsch KG type ZM1 electric mill (Retsch, Haan, Germany) and stored in airtight plastic containers.

2.3. Rumen fluid collection and rumen microbial co-cultures

Incubation techniques and the establishment of the different microbial populations have been described in detail in the previous chapter. Generally spoken, the standard HGT according to Menke et al. (1979) and Menke and Steingass (1988) represented the starting inoculum for all five rumen microbial populations (whole rumen fluid; fungi+protozoa; bacteria+protozoa; protozoa and bacteria+fungi). While whole rumen fluid represents the

population of the standard HGT procedure, in the fungi+protozoa, bacteria+protozoa and protozoa populations parts of the microbes were switched off chemically. This was done using Penicillin-G plus Streptomycin sulphate to suppress bacteria (fungi+protozoa), clotrimazole to suppress fungi (bacteria+protozoa) or a combination to suppress both (protozoa). The bacteria+fungi population was created by reducing protozoa numbers by about one order of magnitude via centrifugation (200 x *g* for 3 min at room temperature) (Mendoza et al., 1995). Supernatant, considerably reduced in protozoa was used as a source of microbes for the incubation inoculum.

All the syringes were incubated in the HGT incubator and gas production was recorded at 4, 8, 12 and 24 h; incubated contents were taken from these incubations for ammonium analysis. Samples for SCFA analysis were taken at 24 h. For each measure, sample size was n = 4 (two runs on different days contributing two samples each).

2.4. Preparation of antibiotic and fungicide solutions

One millilitre of antibiotic stock solution contained 60000 U (37.5 mg) of penicillin-G (Sigma-Aldrich, St. Louis, USA) and 3900 U (5.34 mg) of streptomycin sulphate (Sigma-Aldrich, St. Louis, USA). One millilitre of the stock solution was added to each fermentation syringe (total volume, 30 mL). Clotrimazole (Sigma-Aldrich, St. Louis, USA) was used at the final concentration of 0.9 mg/mL to inhibit the fungal growth. The antibiotic and fungicide solutions were prepared by dissolving in Millipore water.

2.5. Chemical analysis

2.5.1. Ammonia-N

Ammonia-nitrogen (NH_3-N) analysis of incubated residues was carried out by using Kjeldahl method but excluding the digestion step to avoid any protein/peptide digestion. A trapped solution was prepared by applying 25 mL of 0.05 M H_2SO_4 and indicator H_3BO_3 in a small flask. A volume of 5 mL of 1 M NaOH was added into Kjeldahl tube (Sartorius® Precisa 404 M SCS) containing the sample before distillation. Distillation was carried out by using distillation equipment (Gerhardt, Königswinter, Germany) running for approximately 3 min for each sample. The titration was conducted by using 0.05 M NaOH. Point of the titration acquired was indicated by the change in indicator colour from purple to light green (greenish). The volume of 1N NaOH used for titration of the samples was recorded and used for calculation of the NH_3-N content in the samples. Prior to sample titration, blanks titrations

were also measured and as well as the titration of standard 0.05 M H_2SO_4 (10 mL of 0.05 M H_2SO_4 standard volumetric solution AppliChem) to obtain NaOH titer. Finally ammonium concentration was calculated from ammonia-N.

2.5.2. Short chain fatty acids

Short chain fatty acid (SCFA) analysis of 24 h incubated residues was conducted by using gas chromatography (Autosystem XL with autosampler; Perkin Elmer, Waltham, USA). Samples (1.5 mL) were prepared by mixing with 0.1 mL of formic acid. Gas chromatography assembly consisted of following parts: Autosystem with autosampler, piller (length 25 m and diameter 0.32 mm, film thickness 0.21 µm), flame ionisation detector, injector (split ratio 1:50, split bottom 47 L/min, Injection volume 1 µl), gas carrier (Helium gas electric carrier 1 bar, 80 kPa pressure), temperature (Injector 250°C, detector 260°C), heat programme (102°C → 130°C, (8°C /min) and detector gas (H_2 flow 31 mL/min; O_2 flow 400 mL/min).

No results on SCFA concentrations can be given for the 90:10 and 90:10BC diet (spoilage of samples).

2.6. Statistical analysis

For statistical analysis of GP and NH_4, the mean of 4, 8, 12 and 24 h values was used. Analysis of the influence of the factors on the dependant variables GP, SCFA and NH_4 was performed using the statistical model given below including the main effects of ration, MP, and their interaction:

$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + \varepsilon_{ijk}$

where

Y_{ijk} = is the observed response;

μ = is the population constant and is common to all observations;

α_i = is the effect of ration i; i = 1-7;

β_j = is the effect of MP j; j = 1-5;

$(\alpha \times \beta)_{ij}$ = is the effect of interaction between ration i and MP j;

ε_{ijk} = is the residual error

For mean comparisons between rations, polynomial orthogonal contrasts (linear, quadratic and cubic) were done for the five rations without BC; the higher order polynomial contrast was always chosen to better fit the model, according to Abdelqader et al. (2009). Linear

orthogonal contrasts were done to evaluate the effect of BC in the rations of rumen fluid donor animals. Linear orthogonal contrasts were also applied in mean comparisons of MP. For GP and SCFA these were whole rumen fluid vs. MP without bacteria, whole rumen fluid vs. MP with bacteria, MP with bacteria vs. MP without bacteria and bacteria+protozoa vs bacteria+fungi; for NH_4^+, they were whole rumen fluid vs. without protozoa (bacteria+fungi), whole rumen fluid vs. with protozoa (bacteria+protozoa, fungi+protozoa, protozoa), with protozoa vs. without protozoa and bacteria+protozoa vs. bacteria+fungi. In the presence of significant interaction, contrasts were done within factor levels (individual diets or MP). All statistical analyses for SCFA were run without the rations 90:10 and 90:10BC (no values available, as stated above).

The general linear model procedure of SAS was used for ANOVA, and all further calculations were done using SAS (SAS, 2007). The standard level of significance for the effects was $P<0.05$. Effects with P-values of $0.1>P>0.05$ are considered as trends. P-values less than 0.0001 are expressed as <0.001 rather than the actual value.

3. Results

3.1. Gas production

Figure 7 shows the cumulative GP at the different fermentation times for the different MP for the 50:50 ration. The mean GP (mL/200 mg dry matter) across different rations and MP is presented in Table 20. Gas production was 33.0 mL with whole rumen fluid and 31.7 mL with bacteria+fungi, 24.3 mL for bacteria+protozoa and 15.9 mL for fungi+protozoa and 15.1 mL for protozoa (Table 19). ANOVA results revealed that gas production was affected by both ration ($P<0.001$) and MP ($P<0.001$), with interaction between the factors ($P<0.001$). In mean comparisons (Table 20), polynomial contrasts confirmed a linear increasing effect of the concentrate proportion on GP ($P<0.05$) for all MP, except for fungi+protozoa, where a quadratic effect with higher values at intermediate levels was present in addition. Among the MP, whole rumen fluid and the other populations with bacteria (bacteria+protozoa and bacteria+fungi) always produced more gas than MP without bacteria. Whole rumen fluid produced more gas than bacteria+protozoa and bacteria+fungi (the other populations with bacteria) in four of the seven rations. Bacteria+fungi produced more gas than bacteria+protozoa in five out of seven rations. No effect of BC in sheep rations was apparent in the data ($P = 0.402 - 0.962$).

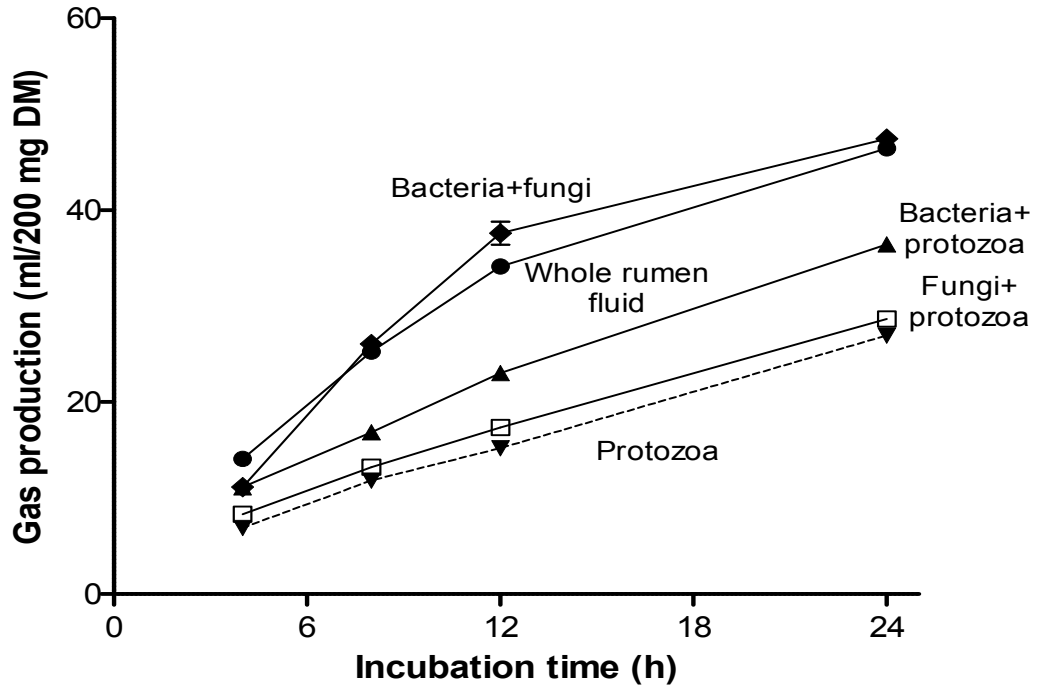

Fig. 7. Gas production of different microbial populations (ration 50:50 concentrate: roughage).

Table 19: Gas and ammonium production with different rations and rumen microbial populations (MP) (mean of 4, 8, 12 and 24 h incubations).

		Gas production (mL/200 DM)	Ammonium production (mmol/L)
Ration (C:R)	10:90	16.8	12.3
	30:70	20.4	12.4
	50:50	22.9	12.7
	70:30BC	28.3	15.1
	70:30	26.0	15.0
	90:10BC	25.9	12.7
	90:10	27.8	13.6
MP	Whole rumen fluid	33.0	12.0
	Fungi+protozoa	15.9	14.3
	Bacteria+protozoa	24.3	14.8
	Protozoa	15.0	14.8
	Bacteria+fungi	31.7	11.0
SEM		0.43	0.11
Significance (P-value)			
Ration		0.001	0.001
MP		0.001	0.001
Ration x MP		0.001	0.001

MP, microbial population; DM, dry matter; C:R, concentrate: roughage; BC, sodium bicarbonate.

Table 20**:** Least squares means for gas production with different diets and rumen microbial populations (24 h incubations).

	Gas production (ml/200 mg DM)									
	Microbial populations						Contrast (*P- value*)			
Ration (C:R)	WRF	F+P	B+P	P	B+F	*SEM*	WRF vs without bacteria	WRF vs with bacteria	B+P vs B+F	With bacteria vs without bacteria
10:90	22.1	10.8	17.6	12.5	20.8	0.67	0.001	0.254	0.263	0.001
30:70	27.7	14.3	19.0	14.9	25.8	0.84	0.001	0.035	0.019	0.001
50:50	30.0	16.9	21.9	15.2	30.6	0.96	0.001	0.136	0.003	0.001
70:30BC	35.5	21.4	31.5	16.5	36.8	1.17	0.001	0.603	0.072	0.001
70:30	36.5	18.5	23.7	15.6	35.8	1.21	0.001	0.009	0.001	0.001
90:10BC	39.3	14.7	26.1	13.6	35.6	1.34	0.001	0.001	0.001	0.001
90: 10	40.1	14.7	30.5	16.9	36.7	1.37	0.001	0.010	0.032	0.001
SEM	1.02	0.52	0.87	0.48	1.07					
	Contrast (*P- value*)									
Linear	0.001	0.013	0.001	0.022	0.001					
Quadratic	0.339	0.001	0.522	0.533	0.092					
Cubic	0.887	0.857	0.508	0.378	0.955					
BC vs no BC	0.663	0.483	0.402	0.572	0.962					
70 vs 90	0.069	0.011	0.731	0.701	0.953					
70+90BC vs 70BC+90	0.050	0.480	0.003	0.307	0.607					

WRF: whole rumen fluid = control; F+P: Fungi+protozoa; B+P: bacteria+fungi; P: Protozoa; B+F: Bacteria+fungi; DM, dry matter.

C:R, concentrate: roughage; BC, sodium bicarbonate.

3.2. Ammonium production

Figure 8 shows the development of ammonium over time for the different MP at the 50:50 ration. Both ration (P<0.001) and MP (P < 0.001) had an effect in the ANOVA for ammonium production; interaction between the factors was present (P<0.001) (Table 19). The populations with protozoa (bacteria+protozoa, fungi+protozoa, protozoa) had higher values than the one without (bacteria+fungi), and than the control (whole rumen fluid). For five of seven rations, whole rumen fluid had higher values than bacteria+fungi. Bacteria+protozoa had higher values than bacteria+fungi (except for ration 30:70) (Table 21). Concerning the effect of ration on ammonium, a linear effect was present for all MP, in some cases combined with either a quadratic (protozoa) or cubic effect (fungi+protozoa; bacteria+protozoa), or both (bacteria+fungi). Little effect of an addition of BC to sheep rations on ammonium levels was visible (P<0.001 only for the protozoa MP).

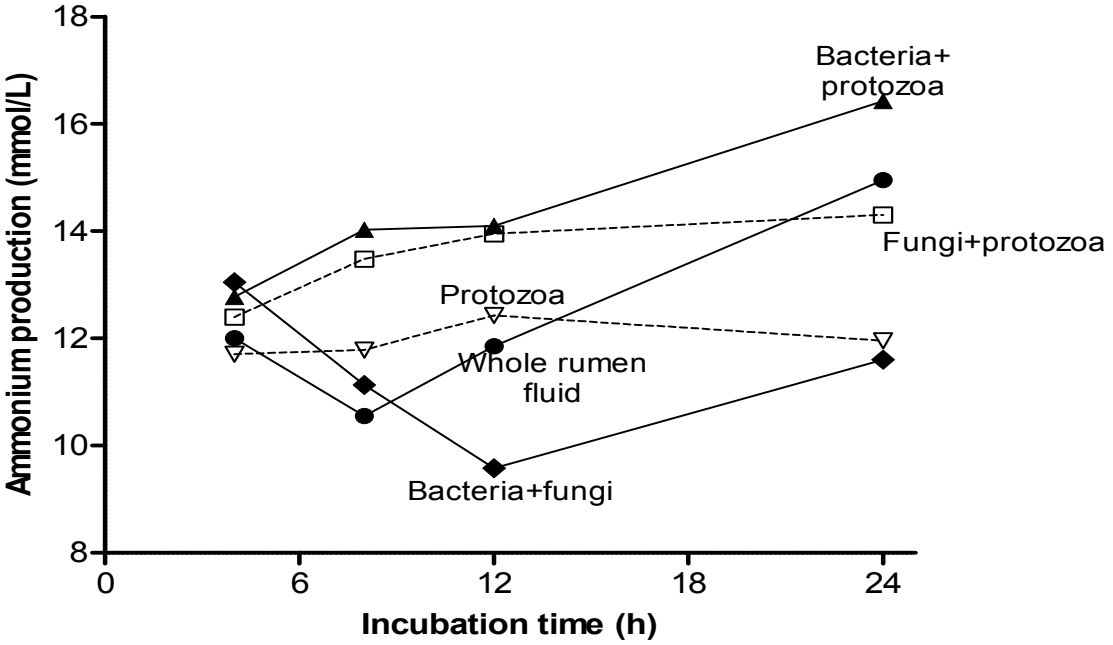

Fig. 8. Ammonium production by different microbial populations (ration 50:50 concentrate: roughage).

Table 21: Least squares means for ammonium production with different rations and rumen microbial populations (24 h incubations).

	Ammonium production (mmol/L)						Contrast (*P-value*)			
	Microbial populations									
Ration (C:R)	WRF	F+P	B+P	P	B+F	*SEM*	WRF vs with protozoa	WRF vs B+F	B+P vs B+F	WRF and with protozoa vs B+F
10:90	10.8	12.3	14.7	14.3	9.5	0.28	0.001	0.008	0.001	0.001
30:70	11.1	11.4	12.0	15.7	11.8	0.23	0.001	0.127	0.747	0.745
50:50	12.3	13.5	14.3	12.0	11.3	0.19	0.024	0.046	0.001	0.001
70:30BC	13.7	17.7	17.2	15.4	11.5	0.31	0.001	0.001	0.001	0.001
70:30	12.5	16.4	17.5	17.0	11.3	0.33	0.001	0.013	0.001	0.001
90:10BC	11.5	13.5	14.0	13.8	10.7	0.22	0.001	0.112	0.001	0.001
90:10	12.1	15.5	13.7	15.6	11.0	0.25	0.001	0.013	0.001	0.001
SEM	0.17	0.23	0.22	0.17	0.16					
	Contrast (*P- value*)									
Linear	0.001	0.001	0.001	0.001	0.053					
Quadratic	0.060	0.688	0.312	0.001	0.001					
Cubic	0.141	0.001	0.001	0.101	0.042					
BC vs no BC	0.415	0.376	0.951	0.001	0.979					
70 vs 90	0.002	0.001	0.001	0.001	0.098					
70+90 BC vs 70 BC+90	0.016	0.001	0.441	0.887	0.530					

WRF: whole rumen fluid = control; F+P: Fungi+protozoa; B+P: bacteria+fungi; P: Protozoa; B+F: Bacteria+fungi; C:R, concentrate:roughage; BC, Sodium bicarbonate

3.3. Short chain fatty acids

For whole rumen fluid, the average values for acetate, propionate and n-butyrate were 14.1, 5.3 and 2.7 mmol/l, with a resulting acetate:propionate molar ratio of 2.7. In the ANOVA, acetate concentration was influenced by ration (P=0.006) and MP (P<0.001), while no interaction between the factors was present (P=0.281) (Table 22). In the MP without bacteria, less acetate was found than in whole rumen fluid (P=0.001) and in the other MP with bacteria (P=0.002). Acetate production increased linearly with concentrate proportion (P=0.001).

For propionate, ration (P=0.001) and MP (P=0.001) both had an effect, and interaction between the factors was also present (P=0.007) (Table 22). Populations without bacteria had a lower propionate level than populations with bacteria and whole rumen fluid (Table 23). A simple linear increasing effect was only present for bacteria+fungi (P=0.001).

The n-butyrate production was influenced by diet (P<0.001) and MP (P<0.001), with interaction between the factors being present (P=0.002) (Table 24). The MP with bacteria had higher n-butyrate concentrations than those without (P=0.035). Little effect was detectable for an addition of BC to sheep diets for the different SCFA (only for propionate with whole rumen fluid; P=0.009).

Concerning the acetate:propionate ratios, there was a significant effect of ration (P=0.003) and MP (P=0.001), whereas no significant interaction was present between the factors (P=0.151). In the MP without bacteria, acetate:propionate ratio was lower than in whole rumen fluid (P=0.001) and MP with bacteria (bacteria+protozoa, bacteria+fungi) (P=0.001). Bacteria+protozoa also had higher values than bacteria+fungi (P=0.008).

Table 22: Short chain fatty acids (SCFA) production with different rations and microbial populations (MP) (24 h incubations).

		SCFA (mmol/L)			A:P ratio
		Acetate	Propionate	n-Butyrate	
Ration (C:R)	10:90	10.9	3.3	1.3	4.7
	30:70	12.2	3.2	2.1	5.7
	50:50	12.3	3.0	3.0	7.9
	70:30 BC	13.0	4.4	3.3	6.3
	70:30	14.8	4.7	2.9	7.3
MP	Whole rumen fluid	14.1	5.3	2.7	2.7
	Fungi+protozoa	11.1	1.3	2.6	9.2
	Bacteria+protozoa	14.5	4.0	2.3	4.5
	Protozoa	9.3	0.7	1.8	13.4
	Bacteria+fungi	14.3	7.3	3.1	2.1
SEM		0.42	0.32	0.12	0.52
Significance (P-value)					
Ration		0.006	0.001	0.001	0.003
MP		0.001	0.001	0.001	0.001
Ration x MP		0.281	0.007	0.002	0.151
		Contrast (P-value)			
Ration	Linear	0.001			0.583
	Quadratic	0.537			0.847
	Cubic	0.269			0.795
	BC vs no BC	0.063			0.674
MP	Whole rumen fluid vs without bacteria	0.001			0.001
	Whole rumen fluid vs with bacteria	0.680			0.417
	Bacteria+protozoa vs bacteria+fungi	0.867			0.008
	With bacteria vs without bacteria	0.001			0.001

Acetate ratio propionate; Concentrate:roughage; Sodium bicarbonate; BC vs no BC = 70:30 + 90:10 vs 70:30 BC + 90:10 BC; 70 vs 90 = 70:30 + 70:30 BC vs 90:10 + 90:10 BC; 70+90 BC vs 70 BC + 90 = 70:30 + 90:10 BC vs 70:30 BC + 90.

Table 23: Least square means for propionate production with different rations and rumen microbial populations (MP).

Ration (C:R)	Propionate production (mmol/L) MP						Contrast (*P-value*)			
	WRF	F+P	B+P	P	B+F	*SEM*	WRF vs without bacteria	WRF vs with bacteria	B+P vs B+F	With bacteria vs without bacteria
10:90	5.3	1.4	3.9	0.8	5.3	0.48	0.001	0.493	0.168	0.001
30:70	4.2	1.1	3.9	1.2	5.9	0.60	0.005	0.430	0.044	0.001
50:50	4.2	1.1	2.8	0.6	6.1	0.51	0.001	0.786	0.001	0.001
70:30 BC	5.1	1.5	6.0	0.5	9.0	0.84	0.001	0.007	0.004	0.001
70:30	7.8	1.3	3.5	0.7	10.2	0.95	0.001	0.290	0.001	0.001
SEM	0.42	0.10	0.39	0.07	0.61					
	Contrast (*P- value*)									
Linear	0.020	0.049	0.560	0.052	0.003					
Quadratic	0.033	0.052	0.232	0.849	0.224					
Cubic	0.007	0.324	0.064	0.046	0.347					
BC vs no BC	0.008	0.901	0.015	0.863	0.212					

WRF: whole rumen fluid = control; F+P: Fungi+protozoa; B+P: bacteria+fungi; P: Protozoa; B+F: Bacteria+fungi; Concentrate:roughage; Sodium bicarbonate; BC vs no BC = 70:30 + 90:10 vs 70:30 BC + 90:10 BC; 70 vs 90 = 70:30 + 70:30 BC vs 90:10 + 90:10 BC; 70+90 BC vs 70 BC + 90 = 70:30 + 90:10 BC vs 70:30 BC + 90.

Table 24: Least square means for n-butyrate production with different rations and rumen microbial populations (MP).

	n-butyrate production (mmol/L)						Contrast (*P-value*)			
	MP									
Ration (C:R)	WRF	F+P	B+P	P	B+F	*SEM*	WRF vs without bacteria	WRF vs with bacteria	B+P vs B+F	With bacteria vs without bacteria
10:90	1.9	0.7	1.1	1.0	1.7	0.13	0.047	0.308	0.269	0.179
30:70	2.2	1.9	2.0	2.1	2.2	0.11	0.778	0.824	0.693	0.910
50:50	3.4	2.3	2.3	1.9	4.7	0.29	0.003	0.880	0.001	0.001
70:30 BC	2.8	4.7	3.4	2.0	3.8	0.30	0.265	0.777	0.463	0.490
70:30	3.1	3.4	2.9	2.2	3.1	0.13	0.554	0.869	0.651	0.604
SEM	0.24	0.36	0.20	0.15	0.29					
	Contrast (*P- value*)									
Linear	0.085	0.001	0.001	0.037	0.001					
Quadratic	0.212	0.001	0.009	0.473	0.012					
Cubic	0.744	0.318	0.170	0.468	0.451					
BC vs no BC	0.581	0.526	0.321	0.748	0.201					

WRF: whole rumen fluid = control; F+P: Fungi+protozoa; B+P: bacteria+fungi; P: Protozoa; B+F: Bacteria+fungi; Concentrate:roughage; Sodium bicarbonate; BC vs no BC = 70:30 + 90:10 vs 70:30 BC + 90:10 BC; 70 vs 90 = 70:30 + 70:30 BC vs 90:10 + 90:10 BC; 70+90 BC vs 70 BC + 90 = 70:30 + 90:10 BC vs 70:30 BC + 90.

Table 25: Influence of different variables on gas production and ammonium contents.

	Gas production
Degradability of feed in general (fermentable energy)	+
For a given degradability:	
Use of feed for energetic purposes	+
Production of microbial mass	-
	Ammonium
Degradation of feed crude protein	+
Digestion of bacterial mass by protozoa	+
Formation of microbial mass	-
Energy available from food	-

4. Discussion

The synthesis of SCFA and microbial protein from the degradation products of feed constituents account for many of the nutrients that are utilized by the host animal and these nutrients arise from the diverse microbial activities present in the rumen (Hungate, 1966; Prins, 1977; Hobson and Wallace, 1982; Russell, 1983). *In vitro* GP, SCFA and ammonium can thus be investigated to study microbial activity (Abou Akkada and El-Shazly, 1964; Van Hoven and Boomker, 1985).

Hohenheim gas test – characteristics of an in vitro system

The Hohenheim gas test in its original intention was meant as a tool to estimate organic matter digestibility (and therefore energy content) of ruminant feeds (Menke et al., 1979). Correspondingly, the major nutritional limitation to the microbial population is energy, since other nutrients like N (as NH_4^+) are supplemented in amounts safely assuring 24 h supply to microbes even if highly digestible samples are incubated. Since its introduction, the HGT experienced many extensions (for some general review see Blümmel et al., 1997; Getachew et al., 1998), such as protein value (utilizable crude protein at the small intestine - uCP) (Steingaß et al., 2001; Leberl et al., 2007), investigations on methane development (Soliva et al., 2003), effects of secondary plant compounds (Khazaal et al., 1994; Makkar et al., 1995) or metabolism of mycotoxins in the rumen (Blank et al., 2002).

Among the advantages of the HGT making the system particularly attractive for questions involving changes in MP is its capacity to keep protozoa numbers at a high level during normal durations of fermentation (e.g. 24 h). This is in contrast to some continuous culture approaches, where protozoa can have some tendency to be washed out (Czerkawski and Breckenridge, 1979; Williams and Coleman, 1997). Besides this, also the acetate to

propionate ratios measured in the system underline the general proximity of the standard MP in the HGT (whole rumen fluid) to a native rumen population (e.g. 2.6-3.7 according to Kirchgeßner, 2004). Significant deviation in other *in vitro* approaches in this respect indicates more significant shifts from native rumen conditions in important characteristics.

When using *in vitro* incubation to study the influence of particular groups of rumen organisms, several points deserve consideration. Among them is that depletion cannot be considered as being complete in most cases. In this study this is obvious for bacteria+fungi, where physical depletion of protozoa led to a decrease by one order of magnitude, but not to complete defaunation. Penicillin is being described to work mainly on growing, but to a far lesser extent on non-growing cells (Schlegel, 1992), just as clotrimazole does work on active fungal cells, but not or much less on spores. Depending on the generation time of the respective microbial group, approximately 12-24 h in protozoa, but only about 30 min in bacteria (Van Soest, 1994; Williams and Coleman, 1997), some limited recovery of the inhibited microbial group can not be excluded completely. It was tried to tackle this by applying the respective antibiotics at two consecutive time intervals for the MP with particularly short generation time (bacteria). A combination of penicillin and streptomycin has regularly been used in studies dependant on depletion of the bacterial community (e.g. Dehority and Tirabasso, 2000; Lee et al., 2000). Via its negative influence on bacterial cell wall formation, penicillin is known to inhibit mainly Gram-positive bacteria but also many Gram-negatives; streptomycin inhibits further Gram-negative bacteria, complementary to those affected by penicillin (Schlegel, 1992). Clotrimazole is known as a broad-spectrum antifungal, its major mode of action being the inhibition of synthesis of an important component of fungal cell membranes (ergosterol); however, it is also known to work on some gram-positive bacteria.

In any evaluation of ruminal metabolism, the interpretation of the measured variables will include some ambiguities. In our example, GP and ammonium can be influenced by several factors, as outlined in Table 25, so any interpretation (especially of ammonium) necessarily includes some assumptions. For both variables measures were done at four different times. The later evaluation of data was done on the level of the least squares mean of these 4 values, representative for both, the total amounts present and formation rate.

4.1. Gas production and ammonium

4.1.1. Influence of MP and ration

In general, the influence of ration on GP was as expected; for the MP whole rumen fluid (= standard HGT inoculum), each increase in concentrate proportion by 200 g/kg led to a corresponding increase of gas production by 4.5 mL/200 mg DM for the values as given in Table 20. The effect of concentrate level on gas production was assumed to be one of the most predictable in this study, given that the HGT represents an *in vitro* system built to evaluate differences in OM digestibility in feeds. Indeed, four out of five possible polynomial contrasts were linear as can be expected, one of the more severely changed populations (fungi+protozoa) being the only exception (with an additional quadratic effect). In general, it is getting very clear from the data that a lack of bacteria has the most important (decreasing) effect on GP. In the case of the bacteria-depleted populations, this is most likely due to a corresponding decrease in digestive capacity. Considering the overall literature on the role of different microbial groups on ruminal metabolism, most focus is on fiber (e.g. Yoder et al., 1966; Chesson and Forsberg, 1997 (for some review); Lee et al., 2000; Zhang et al., 2007) and protein (Wallace et al., 1997) digestion. The focus on fiber digestion kind of implies that this is considered a particularly sophisticated feature among ruminal microbes, where more significant differences can be expected than in other metabolic characteristics, like e.g. capacity to digest starch. Accordingly, one could expect in this study the difference between MP to be more pronounced in diets high in roughage compared to those high in concentrate. In fact, there was interaction between the factors MP and ration. However, when evaluating the data (e.g. by dividing the GP of whole rumen fluid by that of a depleted MP like fungi+protozoa for the individual diets), data does not indicate any systematic change of this value with concentrate level, and therefore no systematic change of the effect of MP with concentrate level.

4.1.2. Bacteria+protozoa vs. bacteria+fungi

From the comparison of bacteria+protozoa and bacteria+fungi, there is some indication that bacteria+protozoa yield lower GP values. Interestingly, in other characteristics depending in some way on “metabolic activity” of the MP like OTA degradation, no clear difference between these MP was obvious (see previous chapter). If at all, bacteria+protozoa showed some tendency for higher OTA-degrading activity. Differences in the final destination of

substrate degraded have been described on different occasions for ruminal digestion. The partitioning factor (Blümmel et al., 1999b), calculated as (substrate actually degraded/GP), differentiates between conditions (in their study different feeds) where most of the substrate degraded is fixed as microbial mass (less GP) or is degraded to the level of SCFA (more GP). However, a first check of this explanation (less GP, but more microbial mass production in bacteria+protozoa compared to bacteria+fungi) is hampered by the fact that higher ammonium occurred in the bacteria+protozoa population, implying less fixation of N (as microbial mass) in this population (see section below). However, in this respect it could be speculated that in being protozoa depleted, bacteria+fungi may represent a particular case in this study, since protozoa have been shown by many studies to increase NH_4^+ levels considerably (e.g. Warner, 1956) – a result that was largely confirmed by this study.

4.1.3. Ammonium contents – effect of protozoa

As a background for any interpretation of ammonium values, the interrelationships as assumed for the estimation of uCP from ammonium contents in the modified HGT (Steingaß et al., 2001) need to be reminded: Changes in ammonium levels give an indication for changes of the sum of newly synthezised microbial CPn and undegraded food CP (= utilizable CP at the duodenum) (GfE, 2001). The higher ammonium concentration in MP with protozoa are generally explained by a significant increase of turnover of ruminal N by this microbial group. The predation and subsequent digestion of bacteria and the corresponding release of ammonium as a product of protein and amino acid digestion are considered to be the major reasons for this. In general, major processes taking influence on ammonium levels are degradation of food CP (more ammonium), formation of bacterial CP by the use of ammonium (less ammonium) and finally also digestion of microbial CP by protozoa (more ammonium). Particularly in closed systems as the HGT, which necessarily become energy depleted at some point, digestion of microbial mass by protozoa and corresponding release of ammonium from microbial protein can be considered the final fate of N at late stages of fermentation.

4.2. Short chain fatty acids

As it represents the basic principle of the HGT (and any other *in vitro* gas production system), total SCFA production is highly correlated with GP; in the HGT, about 500mL/L represent the waste gases CO_2 and CH_4, and the other 500 ml/L come from CO_2 from the buffering reaction (Blümmel et al., 1999a). It was therefore surprising to see that only for acetate clear evidence for a linear effect of ration on SCFA production was present. It should be added here that two practical circumstances may have made the statistical analysis of such pattern more difficult than for GP (where a linear effect was present in most cases): Firstly, due to the loss of the diets highest in concentrate levels (90:10 and 90:10 BC) for SCFA analysis, only four instead of five rations could be used for polynomial contrasts; in addition, for SCFA only 24 h samples were taken, while the GP values presented in Table 20 and 21 represent means of the GP of 4, 8, 12 and 24 h. The latter value thus integrates both total degradability and degradation rate of the substrate; differences due to degradation rate will be less relevant in SCFA than in GP evaluation, therefore. However, whatever the explanation is, the lack of a clear linear effect of ration on propionate and butyrate is considered as surprising.

One of the most prominent results from SCFA analysis were the very low propionate contents in the populations without bacteria (fungi+protozoa and protozoa; 1.3 and 0.7 mmol/L in table 22) compared to those including bacteria (whole rumen fluid; bacteria+protozoa; bacteria+fungi; 5.3, 4.0 and 7.3 mmol/L, respectively). The low propionate levels become understandable from the fact that protozoa have acetate and butyrate as major fermentation products (Williams and Coleman, 1997), although Ellis et al. (1991) also report minimal (~0.01 mole/mol of acetate) amounts of propionate in *Dasytricha* incubations, and ruminal fungi mainly produce acetate and lactate (Orpin and Joblin, 1997).

While most information on acetate:propionate ratios focuses on influence of feeds on this fermentation characteristic, in our approach, both diet and MP had a large influence. Average acetate:propionate ratios of 2.7 (whole rumen fluid), 4.5 (bacteria+protozoa) and 2.1 (bacteria+fungi) were measured, compared to 9.2 (fungi+protozoa) and 13.4 (protozoa) in MP without bacteria. The range of average actetate:propionate ratios among rations was much narrower (4.7-7.9). In vivo, ruminal acetate:propionate ratios of dairy cattle are reported to range between 2.6 (on pasture) and 3.7 (during winter feeding) (Kirchgeßner, 2004), or between 3.6 (moderate roughage diet, 600 g/kg DM concentrate) and 1.2 (low roughage ration, 900 g/kg DM concentrate) (Sutton et al., 1988).

4.3. Influence of $NaHCO_3$

Little effect of BC addition on results was found in this study. Since no BC was added to the *in vitro* incubations (HFT buffer alone guarantees sufficient buffering capacity), any effect would have been based on shifts in the MP of donor animals. The lack of any effect implies that shifts in concentrate:roughage ratio in the range of this study did not lead to serious changes in the ruminal MP during the adaptation period. While a concentrate level of 90% as applied in this trial can be considered as very high, total amounts of feeds given were only rather low (only as appropriate for animals with maintenance requirements), confirming experience that consequences of low roughage and thus, high concentrate intakes are much more relevant at the high intakes of producing animals.

5. Conclusions

The microbial community of the rumen represents a complex community, in many aspects equivalent to a complete ecosystem. In particular, protozoa can be considered to add further trophic levels to this community. By the measurement of some variables integrating processes of energy (largely carbohydrate) and N metabolism, no global and in every aspect final conclusions on the diverse interrelationships and metabolism of ruminal organisms should be expected. Still, some results of this study strongly confirm established views in rumen microbiology, while others contribute to open questions in this field, warranting further attention. Among the first group are the following statements:

- Microbial populations without bacteria show considerably less organic matter degradation capacity;
- Presence of protozoa increases ammonium levels considerably;
- In bacteria depleted populations, little propionate is produced.

The following can be considered to fit into the second group:

- Gas production was higher in bacteria+fungi compared to bacteria+protozoa; why is that?
- No influence of ration (concentrate level) on the effect of MP on GP was detected.

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6 GENERAL CONCLUSIONS

6 General conclusions

While several studies on OTA in ruminants have improved our knowledge significantly over the last three decades, some knowledge gaps still appeared to us in the process of summing up results. Based on literature survey, the incidence of OTA contamination is highest in concentrates, while any evidence for the occurrence in forages is limited. Since monogastric animals generally are fed on concentrate-only diets, a lower daily intake of OTA can be presumed for a ruminant compared to monogastric animals. Considerable concentrations of OTA can occur in ruminant diets, when diets rich in concentrates or forages contaminated with OTA are fed.

Ruminants are relatively resistant to OTA. They can degrade 12 mg OTA/kg feed based on *in vitro* studies (Hult et al., 1976). The capacity of dairy cows to degrade OTA is in the range of 33-72 mg/d and 3-7 mg OTA/d is degradable by sheep (Müller, 1995). The common point of view that OTA is degraded completely by an active rumen microbial population under all circumstances does not always hold true based on evidence from improved analytical procedures and equipment. Practical relevance of OTA in ruminants would be information of systemic occurrence levels and transfer into milk in ruminants fed at the high feeding levels typical for lactating animals for sufficiently long period at praxis-relevant OTA-concentrations. This will further assure judgements of the potential of OTA to induce harmful effects in ruminants.

The present *in vitro* results indicate that bacteria are of highest relevance for OTA degradation while the role of protozoa in OTA degradation appears to be less important than previously reported. Moderate level of C:R ratios is appropriate for OTA degradation. Regarding OTA degradation, the role of rumen fungi is not clear. The half-lives of OTA for the whole rumen fluid population correspond well with *in vivo* results. The good survival of protozoa in HGT represents a secondary result. The rumen microbes excluding bacteria are much less capable to degrade organic matter. However, ammonium contents are considerably increased in the presence of protozoa. Therefore it is concluded that variations in OTA and organic matter degradation were attributed to relative proportion of concentrate and roughage in the ration and a particular microbial combination in rumen fluid.

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	Islamabad, Pakistan
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